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(54) Title: CLONED GENOMES OF INFECTIOUS HEPATITIS C VIRUSES AND USES THEREOF			
(57) Abstract The present invention discloses nucleic acid sequences which encode infectious hepatitis C viruses and the use of these sequences, and polypeptides encoded by all or part of these sequences, in the development of vaccines and diagnostics for HCV and in the development of screening assays for the identification of antiviral agents for HCV.			

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Title Of Invention

Cloned Genomes Of Infectious
Hepatitis C Viruses And Uses Thereof

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This application claims the benefit of U.S.
Provisional Application No. 60/053,062 filed July 18,
1997.

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Field Of Invention

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The present invention relates to molecular
approaches to the production of nucleic acid sequences
which comprise the genome of infectious hepatitis C
viruses. In particular, the invention provides nucleic
acid sequences which comprise the genomes of infectious
hepatitis C viruses of genotype 1a and 1b strains. The
invention therefore relates to the use of these sequences,
and polypeptides encoded by all or part of these
sequences, in the development of vaccines and diagnostic
assays for HCV and in the development of screening assays
for the identification of antiviral agents for HCV.

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Background Of Invention

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Hepatitis C virus (HCV) has a positive-sense
single-strand RNA genome and is a member of the virus
family *Flaviviridae* (Choo et al., 1991; Rice, 1996). As
for all positive-stranded RNA viruses, the genome of HCV
functions as mRNA from which all viral proteins necessary
for propagation are translated.

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The viral genome of HCV is approximately 9600
nucleotides (nts) and consists of a highly conserved 5'
untranslated region (UTR), a single long open reading

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frame (ORF) of approximately 9,000 nts and a complex 3'
UTR. The 5' UTR contains an internal ribosomal entry site
(Tsukiyama-Kohara et al., 1992; Honda et al., 1996). The
5 3' UTR consists of a short variable region, a
polypyrimidine tract of variable length and, at the 3'
end, a highly conserved region of approximately 100 nts
(Kolykhalov et al., 1996; Tanaka et al., 1995; Tanaka et
al., 1996; Yamada et al., 1996). The last 46 nucleotides
10 of this conserved region were predicted to form a stable
stem-loop structure thought to be critical for viral
replication (Blight and Rice, 1997; Ito and Lai, 1997;
Tsuchihara et al., 1997). The ORF encodes a large
15 polypeptide precursor that is cleaved into at least 10
proteins by host and viral proteinases (Rice, 1996). The
predicted envelope proteins contain several conserved N-
linked glycosylation sites and cysteine residues (Okamoto
et al., 1992a). The NS3 gene encodes a serine protease
20 and an RNA helicase and the NS5B gene encodes an RNA-
dependent RNA polymerase.

Globally, six major HCV genotypes (genotypes 1-
6) and multiple subtypes (a, b, c, etc.) have been
25 identified (Bukh et al., 1993; Simmonds et al., 1993).
The most divergent HCV isolates differ from each other by
more than 30% over the entire genome (Okamoto et al.,
1992a) and HCV circulates in an infected individual as a
quasispecies of closely related genomes (Bukh et al.,
30 1995; Farci et al., 1997).

At present, more than 80% of individuals
infected with HCV become chronically infected and these
chronically infected individuals have a relatively high
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risk of developing chronic hepatitis, liver cirrhosis and
hepatocellular carcinoma (Hoofnagle, 1997). In the U.S.,
HCV genotypes 1a and 1b constitute the majority of
infections while in many other areas, especially in Europe
5 and Japan, genotype 1b predominates.

The only effective therapy for chronic hepatitis
C, interferon (IFN), induces a sustained response in less
than 25% of treated patients (Fried and Hoofnagle, 1995).
10 Consequently, HCV is currently the most common cause of
end stage liver failure and the reason for about 30% of
liver transplants performed in the U.S. (Hoofnagle, 1997).
In addition, a number of recent studies suggested that the
severity of liver disease and the outcome of therapy may
15 be genotype-dependent (reviewed in Bukh et al., 1997). In
particular, these studies suggested that infection with
HCV genotype 1b was associated with more severe liver
disease (Brechot, 1997) and a poorer response to IFN
20 therapy (Fried and Hoofnagle, 1995). As a result of the
inability to develop a universally effective therapy
against HCV infection, it is estimated that there are
still more than 25,000 new infections yearly in the U.S.
25 (Alter 1997) Moreover, since there is no vaccine for HCV,
HCV remains a serious public health problem.

However, despite the intense interest in the
development of vaccines and therapies for HCV, progress
has been hindered by the absence of a useful cell culture
30 system and the lack of any small animal model for
laboratory study. For example, while replication of HCV
in several cell lines has been reported, such observations
have turned out not to be highly reproducible. In

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addition, the chimpanzee is the only animal model, other
than man, for this disease. Consequently, HCV has been
able to be studied only by using clinical materials
obtained from patients or experimentally infected
5 chimpanzees (an animal model whose availability is very
limited).

However, several researchers have recently
reported the construction of infectious cDNA clones of
10 HCV, the identification of which would permit a more
effective search for susceptible cell lines and facilitate
molecular analysis of the viral genes and their function.
For example, Dash et al., (1997) and Yoo et al., (1995)
15 reported that RNA transcripts from cDNA clones of HCV-1
(genotype 1a) and HCV-N (genotype 1b), respectively,
resulted in viral replication after transfection into
human hepatoma cell lines. Unfortunately, the viability
of these clones was not tested in vivo and concerns were
20 raised about the infectivity of these cDNA clones in vitro
(Fausto, 1997). In addition, both clones did not contain
the terminal 98 conserved nucleotides at the very 3' end
of the UTR.

25 Kolykhalov et al., (1997) and Yanagi et al.
(1997) reported the derivation from HCV strain H77 (which
is genotype 1a) of cDNA clones of HCV that are infectious
for chimpanzees. However, while these infectious clones
will aid in studying HCV replication and pathogenesis and
30 will provide an important tool for development of in vitro
replication and propagation systems, it is important to
have infectious clones of more than one genotype given the
extensive genetic heterogeneity of HCV and the potential

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impact of such heterogeneity on the development of effective therapies and vaccines for HCV.

Summary Of The Invention

5 The present invention relates to nucleic acid sequences which comprise the genome of infectious hepatitis C viruses and in particular, nucleic acid sequences which comprise the genome of infectious
10 hepatitis C viruses of genotype 1a and 1b strains. It is therefore an object of the invention to provide nucleic acid sequences which encode infectious hepatitis C viruses. Such nucleic acid sequences are referred to
15 throughout the application as "infectious nucleic acid sequences".

 For the purposes of this application, nucleic acid sequence refers to RNA, DNA, cDNA or any variant thereof capable of directing host organism synthesis of a
20 hepatitis C virus polypeptide. It is understood that nucleic acid sequence encompasses nucleic acid sequences, which due to degeneracy, encode the same polypeptide sequence as the nucleic acid sequences described herein.

25 The invention also relates to the use of the infectious nucleic acid sequences to produce chimeric genomes consisting of portions of the open reading frames of infectious nucleic acid sequences of other genotypes (including, but not limited to, genotypes 1, 2, 3, 4, 5
30 and 6) and subtypes (including, but not limited to, subtypes 1a, 1b, 2a, 2b, 2c, 3a 4a-4f, 5a and 6a) of HCV. For example infectious nucleic acid sequence of the 1a and 1b strains H77 and HC-J4, respectively, described herein

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can be used to produce chimeras with sequences from the genomes of other strains of HCV from different genotypes or subtypes. Nucleic acid sequences which comprise sequence from the open-reading frames of 2 or more HCV
5 genotypes or subtypes are designated "chimeric nucleic acid sequences".

The invention further relates to mutations of the infectious nucleic acid sequences of the invention
10 where mutation includes, but is not limited to, point mutations, deletions and insertions. In one embodiment, a gene or fragment thereof can be deleted to determine the effect of the deleted gene or genes on the properties of the encoded virus such as its virulence and its ability to
15 replicate. In an alternative embodiment, a mutation may be introduced into the infectious nucleic acid sequences to examine the effect of the mutation on the properties of the virus in the host cell.

The invention also relates to the introduction
20 of mutations or deletions into the infectious nucleic acid sequences in order to produce an attenuated hepatitis C virus suitable for vaccine development.

The invention further relates to the use of the
25 infectious nucleic acid sequences to produce attenuated viruses via passage in vitro or in vivo of the viruses produced by transfection of a host cell with the infectious nucleic acid sequence.

The present invention also relates to the use of
30 the nucleic acid sequences of the invention or fragments thereof in the production of polypeptides where "nucleic acid sequences of the invention" refers to infectious

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nucleic acid sequences, mutations of infectious nucleic
acid sequences, chimeric nucleic acid sequences and
sequences which comprise the genome of attenuated viruses
produced from the infectious nucleic acid sequences of the
5 invention. The polypeptides of the invention, especially
structural polypeptides, can serve as immunogens in the
development of vaccines or as antigens in the development
of diagnostic assays for detecting the presence of HCV in
10 biological samples.

The invention therefore also relates to vaccines
for use in immunizing mammals especially humans against
hepatitis C. In one embodiment, the vaccine comprises one
or more polypeptides made from a nucleic acid sequence of
15 the invention or fragment thereof. In a second
embodiment, the vaccine comprises a hepatitis C virus
produced by transfection of host cells with the nucleic
acid sequences of the invention.

20 The present invention therefore relates to
methods for preventing hepatitis C in a mammal. In one
embodiment the method comprises administering to a mammal
a polypeptide or polypeptides encoded by a nucleic acid
sequence of the invention in an amount effective to induce
25 protective immunity to hepatitis C. In another
embodiment, the method of prevention comprises
administering to a mammal a hepatitis C virus of the
invention in an amount effective to induce protective
30 immunity against hepatitis C.

In yet another embodiment, the method of
protection comprises administering to a mammal a nucleic
acid sequence of the invention or a fragment thereof in an

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amount effective to induce protective immunity against hepatitis C.

The invention also relates to hepatitis C viruses produced by host cells transfected with the nucleic acid sequences of the present invention.

The invention therefore also provides pharmaceutical compositions comprising the nucleic acid sequences of the invention and/or their encoded hepatitis C viruses. The invention further provides pharmaceutical compositions comprising polypeptides encoded by the nucleic acid sequences of the invention or fragments thereof. The pharmaceutical compositions of the invention may be used prophylactically or therapeutically.

The invention also relates to antibodies to the hepatitis C viruses of the invention or their encoded polypeptides and to pharmaceutical compositions comprising these antibodies.

The present invention further relates to polypeptides encoded by the nucleic acid sequences of the invention fragments thereof. In one embodiment, said polypeptide or polypeptides are fully or partially purified from hepatitis C virus produced by cells transfected with nucleic acid sequence of the invention. In another embodiment, the polypeptide or polypeptides are produced recombinantly from a fragment of the nucleic acid sequences of the invention. In yet another embodiment, the polypeptides are chemically synthesized.

The invention also relates to the use of the nucleic acid sequences of the invention to identify cell

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lines capable of supporting the replication of HCV in vitro.

The invention further relates to the use of the nucleic acid sequences of the invention or their encoded proteases (e.g. NS3 protease) to develop screening assays to identify antiviral agents for HCV.

Brief Description Of Figures

Figure 1 shows a strategy for constructing full-length cDNA clones of HCV strain H77. The long PCR products amplified with H1 and H9417R primers were cloned directly into pGEM-9zf(-) after digestion with Not I and Xba I (pH21_I and pH50_I). Next, the 3' UTR was cloned into both pH21_I and pH50_I after digestion with Afl II and Xba I (pH21 and pH50). pH21 was tested for infectivity in a chimpanzee. To improve the efficiency of cloning, we constructed a cassette vector with consensus 5' and 3' termini of H77. This cassette vector (pCV) was obtained by cutting out the BamHI fragment (nts 1358- 7530 of the H77 genome) from pH50, followed by religation. Finally, the long PCR products of H77 amplified with primers H1 and H9417R (H product) or primers A1 and H9417R (A product) were cloned into pCV after digestion with Age I and Afl II or with Pin AI and Bfr I. The latter procedure yielded multiple complete cDNA clones of strain H77 of HCV.

Figure 2 shows the results of gel electrophoresis of long RT-PCR amplicons of the entire ORF of H77 and the transcription mixture of the infectious clone of H77. The complete ORF was amplified by long RT-PCR with the primers H1 or A1 and H9417R from 10⁵ GE of

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5 H77. A total of 10 μ g of the consensus chimeric clone (pCV-H77C) linearized with Xba I was transcribed in a 100 μ l reaction with T7 RNA polymerase. Five μ l of the transcription mixture was analyzed by gel electrophoresis and the remainder of the mixture was injected into a chimpanzee. Lane 1, molecular weight marker ; lane 2, products amplified with primers H1 and H9417R; lane 3, products amplified with primers A1 and H9417R; lane 4, transcription mixture containing the RNA transcripts and linearized clone pCV-H77C (12.5 kb).

Figure 3 is a diagram of the genome organization of HCV strain H77 and the genetic heterogeneity of individual full-length clones compared with the consensus sequence of H77. Solid lines represent aa changes. Dashed lines represent silent mutations. A * in pH21 represents a point mutation at nt 58 in the 5' UTR. In the ORF, the consensus chimeric clone pCV-H77C had 11 nt differences [at positions 1625 (C→T), 2709 (T→C), 3380 (A→G), 3710 (C→T), 3914 (G→A), 4463 (T→C), 5058 (C→T), 5834 (C→T), 6734 (T→C), 7154 (C→T), and 7202 (T→C)] and one aa change (F → L at aa 790) compared with the consensus sequence of H77. This clone was infectious. Clone pH21 and pCV-H11 had 19 nts (7 aa) and 64 nts (21 aa) differences respectively, compared with the consensus sequence of H77. These two clones were not infectious. A single point mutation in the 3' UTR at nucleotide 9406 (G→A) introduced to create an Afl II cleavage site is not shown.

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Figures 4A-4F show the complete nucleotide sequence of a H77C clone produced according to the present invention and Figures 4G-4H show the amino acid sequence encoded by the H77C clone.

5 Figure 5 shows an agarose gel of long RT-PCR amplicons and transcription mixtures. Lanes 1 and 4: Molecular weight marker (*Lambda/HindIII* digest). Lanes 2 and 3: RT-PCR amplicons of the entire ORF of HC-J4. Lane 10 5: pCV-H77C transcription control (Yanagi et al., 1997). Lanes 6, 7, and 8: 1/40 of each transcription mixture of pCV-J4L2S, pCV-J4L4S and pCV-J4L6S, respectively, which was injected into the chimpanzee.

15 Figure 6 shows the strategy utilized for the construction of full-length cDNA clones of HCV strain HC-J4. The long PCR products were cloned as two separate fragments (L and S) into a cassette vector (pCV) with fixed 5' and 3' termini of HCV (Yanagi et al., 1997). 20 Full-length cDNA clones of HC-J4 were obtained by inserting the L fragment from three pCV-J4L clones into three identical pCV-J4S9 clones after digestion with *PinAI* (isoschizomer of *AgeI*) and *BfrI* (isoschizomer of *AflIII*). 25

Figure 7 shows amino acid positions with a quasispecies of HC-J4 in the acute phase plasma pool obtained from an experimentally infected chimpanzee. Cons-p9: consensus amino acid sequence deduced from 30 analysis of nine L fragments and nine S fragments (see Fig. 6). Cons-D: consensus sequence derived from direct sequencing of the PCR product. A, B, C: groups of similar viral species. Dot: amino acid identical to that in Cons-

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p9. Capital letter: amino acid different from that in
Cons-p9. Cons-F: composite consensus amino acid sequence
combining Cons-p9 and Cons-D. Boxed amino acid: different
from that in Cons-F. Shaded amino acid: different from
5 that in all species A sequences. An *: defective ORF due
to a nucleotide deletion (clone L1, aa 1097) or insertion
(clone L7, aa 2770). Diagonal lines: fragments used to
construct the infectious clone.

10 Figure 8 shows comparisons (percent difference)
of nucleotide (nts. 156 - 8935) and predicted amino acid
sequences (aa 1 - 2864) of L clones (species A, B, and C,
this study), HC-J4/91 (Okamoto et al., 1992b) and HC-J4/83
15 (Okamoto et al., 1992b). Differences among species A
sequences and among species B sequences are shaded.

Figure 9 shows UPGMA ("unweighted pair group
method with arithmetic mean") trees of HC-J4/91 (Okamoto
et al., 1992b), HC-J4/83 (Okamoto et al., 1992b), two
20 prototype strains of genotype 1b (HCV-J, Kato et al.,
1990; HCV-BK, Takamizawa et al., 1991), and L clones (this
study).

Figure 10 shows the alignment of the HVR1 and
25 HVR2 amino acid sequences of the E2 sequences of nine L
clones of HC-J4 (species A, B, and C) obtained from an
early acute phase plasma pool of an experimentally
infected chimpanzee compared with the sequences of eight
clones (HC-J4/91-20 through HC-J4/91-27, Okamoto et al.,
30 1992b) derived from the inoculum. Dot: an amino acid
identical to that in the top line. Capital letters: amino
acid different from that in the top line.

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Figure 11 shows the alignment of the 5' UTR and the 3' UTR sequences of infectious clones of genotype 1a (pCV-H77C) and 1b (pCV-J4L6S). Top line: consensus sequence of the indicated strain. Dot: identity with consensus sequence. Capital letter: different from the consensus sequence. Dash: deletion. Underlined: *PinAI* and *BfrI* cleavage site. Numbering corresponds to the HCV sequence of pCV-J4L6S.

Figure 12 shows a comparison of individual full-length cDNA clones of the ORF of HCV strain HC-J4 with the consensus sequence (see Fig. 7). Solid lines: amino acid changes. Dashed lines: silent mutations. Clone pCV-J4L6S was infectious in vivo whereas clones pCV-J4L2S and pCV-J4L4S were not.

Figure 13 shows biochemical (ALT levels) and PCR analyses of a chimpanzee following percutaneous intrahepatic transfection with RNA transcripts of the infectious clone of pCV-J4L2S, pCV-J4L4S and pCV-J4L6S. The ALT serum enzyme levels were measured in units per liter (u/l). For the PCR analysis, "HCV RNA" represented by an open rectangle indicates a serum sample that was negative for HCV after nested PCR; "HCV RNA" represented by a closed rectangle indicates that the serum sample was positive for HCV and HCV GE titer on the right-hand y-axis represents genome equivalents.

Figures 14A-14F show the nucleotide sequence of the infectious clone of genotype 1b strain HC-J4 and Figures 14G-14H show the amino acid sequence encoded by the HC-J4 clone.

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Figure 15 shows the strategy for constructing a chimeric HCV clone designated pH77CV-J4 which contains the nonstructural region of the infectious clone of genotype 1a strain H77 and the structural region of the infectious clone of genotype 1b strain HC-J4.

Figures 16A-16F show the nucleotide sequence of the chimeric 1a/1b clone pH77CV-J4 of Figure 15 and Figures 16G-16H show the amino acid sequence encoded by the chimeric 1a/1b clone.

Figures 17A and 17B show the sequence of the 3' untranslated region remaining in various 3' deletion mutants of the 1a infectious clone pCV-H77C and the strategy utilized in constructing each 3' deletion mutant (Figures 17C-17G).

Of the seven deletion mutants shown, two (pCV-H77C(-98X) and (-42X)) have been constructed and tested for infectivity in chimpanzees (see Figures 17A and 17C) and the other six are to be constructed and tested for infectivity as described in Figures 17D-17G.

Figures 18A and 18B show biochemical (ALT levels), PCR (HCV RNA and HCV GE titer), serological (anti-HCV) and histopathological (Fig. 18B only) analyses of chimpanzees 1494 (Fig. 18A) and 1530 (Fig. 18B) following transfection with the infectious cDNA clone pCV-H77C.

The ALT serum enzyme levels were measured in units per ml (u/l). For the PCR analysis, "HCV RNA" represented by an open rectangle indicates a serum sample that was negative for HCV after nested PCR; "HCV RNA" represented by a closed rectangle indicates that the serum

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sample was positive for HCV; and HCV GE titer on the right-hand y-axis represents genome equivalents.

The bar marked "anti-HCV" indicates samples that were positive for anti-HCV antibodies as determined by commercial assays. The histopathology scores in Figure 18B correspond to no histopathology (O), mild hepatitis (●) and moderate to severe hepatitis (●).

DESCRIPTION OF THE INVENTION

The present invention relates to nucleic acid sequences which comprise the genome of an infectious hepatitis C virus. More specifically, the invention relates to nucleic acid sequences which encode infectious hepatitis C viruses of genotype 1a and 1b strains. In one embodiment, the infectious nucleic acid sequence of the invention has the sequence shown in Figures 4A-4F of this application. In another embodiment, the infectious nucleic acid sequence has the sequence shown in Figures 14A-14F and is contained in a plasmid construct deposited with the American Type Culture Collection (ATCC) on January 26, 1998 and having ATCC accession number 209596.

The invention also relates to "chimeric nucleic acid sequences" where the chimeric nucleic acid sequences consist of open-reading frame sequences taken from infectious nucleic acid sequences of hepatitis C viruses of different genotypes or subtypes.

In one embodiment, the chimeric nucleic acid sequence consists of sequence from the genome of an HCV strain belonging to one genotype or subtype which encodes structural polypeptides and sequence of an HCV strain

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belonging to another genotype strain or subtype which
encodes nonstructural polypeptides. Such chimeras can be
produced by standard techniques of restriction digestion,
PCR amplification and subcloning known to those of
5 ordinary skill in the art.

In a preferred embodiment, the sequence encoding
nonstructural polypeptides is from an infectious nucleic
acid sequence encoding a genotype 1a strain where the
10 construction of a chimeric 1a/1b nucleic acid sequence is
described in Example 9 and the chimeric 1a/1b nucleic acid
sequence is shown in Figures 16A-16F. It is believed that
the construction of such chimeric nucleic acid sequences
will be of importance in studying the growth and virulence
15 properties of hepatitis C virus and in the production of
hepatitis C viruses suitable to confer protection against
multiple genotypes of HCV. For example, one might produce
a "multivalent" vaccine by putting epitopes from several
20 genotypes or subtypes into one clone. Alternatively one
might replace just a single gene from an infectious
sequence with the corresponding gene from the genomic
sequence of a strain from another genotype or subtype or
create a chimeric gene which contains portions of a gene
25 from two genotypes or subtypes. Examples of genes which
could be replaced or which could be made chimeric,
include, but are not limited to, the E1, E2 and NS4 genes.

The invention further relates to mutations of
30 the infectious nucleic acid sequences where "mutations"
includes, but is not limited to, point mutations,
deletions and insertions. Of course, one of ordinary
skill in the art would recognize that the size of the

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insertions would be limited by the ability of the resultant nucleic acid sequence to be properly packaged within the virion. Such mutation could be produced by techniques known to those of skill in the art such as
5 site-directed mutagenesis, fusion PCR, and restriction digestion followed by religation.

In one embodiment, mutagenesis might be undertaken to determine sequences that are important for
10 viral properties such as replication or virulence. For example, one may introduce a mutation into the infectious nucleic acid sequence which eliminates the cleavage site between the NS4A and NS4B polypeptides to examine the effects on viral replication and processing of the
15 polypeptide. Alternatively, one or more of the 3 amino acids encoded by the infectious 1b nucleic acid sequence shown in Figures 14A-14F which differ from the HC-J4 consensus sequence may be back mutated to the
20 corresponding amino acid in the HC-J4 consensus sequence to determine the importance of these three amino acid changes to infectivity or virulence. In yet another embodiment, one or more of the amino acids from the
25 noninfectious 1b clones pCV-J4L2S and pCV-J4L4S which differ from the consensus sequence may be introduced into the infectious 1b sequence shown in Figures 14A-14F.

In yet another example, one may delete all or part of a gene or of the 5' or 3' nontranslated region
30 contained in an infectious nucleic acid sequence and then transfect a host cell (animal or cell culture) with the mutated sequence and measure viral replication in the host by methods known in the art such as RT-PCR. Preferred

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genes include, but are not limited to, the P7, NS4B and NS5A genes. Of course, those of ordinary skill in the art will understand that deletion of part of a gene, preferably the central portion of the gene, may be
5 preferable to deletion of the entire gene in order to conserve the cleavage site boundaries which exist between proteins in the HCV polyprotein and which are necessary for proper processing of the polyprotein.

10 In the alternative, if the transfection is into a host animal such as a chimpanzee, one can monitor the virulence phenotype of the virus produced by transfection of the mutated infectious nucleic acid sequence by methods known in the art such as measurement of liver enzyme
15 levels (alanine aminotransferase (ALT) or isocitrate dehydrogenase (ICD)) or by histopathology of liver biopsies. Thus, mutations of the infectious nucleic acid sequences may be useful in the production of attenuated
20 HCV strains suitable for vaccine use.

The invention also relates to the use of the infectious nucleic acid sequences of the present invention to produce attenuated viral strains via passage in vitro or in vivo of the virus produced by transfection with the
25 infectious nucleic acid sequences.

The present invention therefore relates to the use of the nucleic acid sequences of the invention to identify cell lines capable of supporting the replication
30 of HCV.

In particular, it is contemplated that the mutations of the infectious nucleic acid sequences of the invention and the production of chimeric sequences as
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discussed above may be useful in identifying sequences critical for cell culture adaptation of HCV and hence, may be useful in identifying cell lines capable of supporting HCV replication.

Transfection of tissue culture cells with the nucleic acid sequences of the invention may be done by methods of transfection known in the art such as electroporation, precipitation with DEAE-Dextran or calcium phosphate or liposomes.

In one such embodiment, the method comprises the growing of animal cells, especially human cells, in vitro and transfecting the cells with the nucleic acid of the invention, then determining if the cells show indicia of HCV infection. Such indicia include the detection of viral antigens in the cell, for example, by immunofluorescent procedures well known in the art; the detection of viral polypeptides by Western blotting using antibodies specific therefor; and the detection of newly transcribed viral RNA within the cells via methods such as RT-PCR. The presence of live, infectious virus particles following such tests may also be shown by injection of cell culture medium or cell lysates into healthy, susceptible animals, with subsequent exhibition of the symptoms of HCV infection.

Suitable cells or cell lines for culturing HCV include, but are not limited to, lymphocyte and hepatocyte cell lines known in the art.

Alternatively, primary hepatocytes can be cultured, and then infected with HCV; or, the hepatocyte cultures could be derived from the livers of infected

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chimpanzees. In addition, various immortalization methods known to those of ordinary skill in the art can be used to obtain cell-lines derived from hepatocyte cultures. For example, primary hepatocyte cultures may be fused to a variety of cells to maintain stability.

The present invention further relates to the in vitro and in vivo production of hepatitis C viruses from the nucleic acid sequences of the invention.

In one embodiment, the sequences of the invention can be inserted into an expression vector that functions in eukaryotic cells. Eukaryotic expression vectors suitable for producing high efficiency gene transfer in vivo are well known to those of ordinary skill in the art and include, but are not limited to, plasmids, vaccinia viruses, retroviruses, adenoviruses and adeno-associated viruses.

In another embodiment, the sequences contained in the recombinant expression vector can be transcribed in vitro by methods known to those of ordinary skill in the art in order to produce RNA transcripts which encode the hepatitis C viruses of the invention. The hepatitis C viruses of the invention may then be produced by transfecting cells by methods known to those of ordinary skill in the art with either the in vitro transcription mixture containing the RNA transcripts (see Example 4) or with the recombinant expression vectors containing the nucleic acid sequences described herein.

The present invention also relates to the construction of cassette vectors useful in the cloning of viral genomes wherein said vectors comprise a nucleic acid

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sequence to be cloned, and said vector reading in the correct phase for the expression of the viral nucleic acid to be cloned. Such a cassette vector will, of course, also possess a promoter sequence, advantageously placed upstream of the sequence to be expressed. Cassette vectors according to the present invention are constructed according to the procedure described in Figure 1, for example, starting with plasmid pCV. Of course, the DNA to be inserted into said cassette vector can be derived from any virus, advantageously from HCV, and most advantageously from the H77 strain of HCV. The nucleic acid to be inserted according to the present invention can, for example, contain one or more open reading frames of the virus, for example, HCV. The cassette vectors of the present invention may also contain, optionally, one or more expressible marker genes for expression as an indication of successful transfection and expression of the nucleic acid sequences of the vector. To insure expression, the cassette vectors of the present invention will contain a promoter sequence for binding of the appropriate cellular RNA polymerase, which will depend on the cell into which the vector has been introduced. For example, if the host cell is a bacterial cell, then said promoter will be a bacterial promoter sequence to which the bacterial RNA polymerases will bind.

The hepatitis C viruses produced from the sequences of the invention may be purified or partially purified from the transfected cells by methods known to those of ordinary skill in the art. In a preferred embodiment, the viruses are partially purified prior to

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their use as immunogens in the pharmaceutical compositions and vaccines of the present invention.

The present invention therefore relates to the use of the hepatitis C viruses produced from the nucleic acid sequences of the invention as immunogens in live or killed (e.g., formalin inactivated) vaccines to prevent hepatitis C in a mammal.

In an alternative embodiment, the immunogen of the present invention may be an infectious nucleic acid sequence, a chimeric nucleic acid sequence, or a mutated infectious nucleic acid sequence which encodes a hepatitis C virus. Where the sequence is a cDNA sequence, the cDNAs and their RNA transcripts may be used to transfect a mammal by direct injection into the liver tissue of the mammal as described in the Examples.

Alternatively, direct gene transfer may be accomplished via administration of a eukaryotic expression vector containing a nucleic acid sequence of the invention.

In yet another embodiment, the immunogen may be a polypeptide encoded by the nucleic acid sequences of the invention. The present invention therefore also relates to polypeptides produced from the nucleic acid sequences of the invention or fragments thereof. In one embodiment, polypeptides of the present invention can be recombinantly produced by synthesis from the nucleic acid sequences of the invention or isolated fragments thereof, and purified, or partially purified, from transfected cells using methods already known in the art. In an alternative embodiment, the polypeptides may be purified or partially

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purified from viral particles produced via transfection of
a host cell with the nucleic acid sequences of the
invention. Such polypeptides might, for example, include
either capsid or envelope polypeptides prepared from the
5 sequences of the present invention.

When used as immunogens, the nucleic acid
sequences of the invention, or the polypeptides or viruses
produced therefrom, are preferably partially purified
10 prior to use as immunogens in pharmaceutical compositions
and vaccines of the present invention. When used as a
vaccine, the sequences and the polypeptide and virus
products thereof, can be administered alone or in a
suitable diluent, including, but not limited to, water,
15 saline, or some type of buffered medium. The vaccine
according to the present invention may be administered to
an animal, especially a mammal, and most especially a
human, by a variety of routes, including, but not limited
20 to, intradermally, intramuscularly, subcutaneously, or in
any combination thereof.

Suitable amounts of material to administer for
prophylactic and therapeutic purposes will vary depending
on the route selected and the immunogen (nucleic acid,
25 virus, polypeptide) administered. One skilled in the art
will appreciate that the amounts to be administered for
any particular treatment protocol can be readily
determined without undue experimentation. The vaccines of
30 the present invention may be administered once or
periodically until a suitable titer of anti-HCV antibodies
appear in the blood. For an immunogen consisting of a
nucleic acid sequence, a suitable amount of nucleic acid

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sequence to be used for prophylactic purposes might be expected to fall in the range of from about 100 µg to about 5 mg and most preferably in the range of from about 500 µg to about 2mg. For a polypeptide, a suitable amount to use for prophylactic purposes is preferably 100 ng to 100 µg and for a virus 10^2 to 10^6 infectious doses. Such administration will, of course, occur prior to any sign of HCV infection.

A vaccine of the present invention may be employed in such forms as capsules, liquid solutions, suspensions or elixirs for oral administration, or sterile liquid forms such as solutions or suspensions. Any inert carrier is preferably used, such as saline or phosphate-buffered saline, or any such carrier in which the HCV of the present invention can be suitably suspended. The vaccines may be in the form of single dose preparations or in multi-dose flasks which can be utilized for mass-vaccination programs of both animals and humans. For purposes of using the vaccines of the present invention reference is made to Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa., Osol (Ed.) (1980); and New Trends and Developments in Vaccines, Voller et al. (Eds.), University Park Press, Baltimore, Md. (1978), both of which provide much useful information for preparing and using vaccines. Of course, the polypeptides of the present invention, when used as vaccines, can include, as part of the composition or emulsion, a suitable adjuvant, such as alum (or aluminum hydroxide) when humans are to be vaccinated, to further stimulate production of antibodies by immune cells. When nucleic acids or viruses are used

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for vaccination purposes, other specific adjuvants such as CpG motifs (Krieg, A.K. et al. (1995) and (1996)), may prove useful.

When the nucleic acids, viruses and polypeptides of the present invention are used as vaccines or inocula, they will normally exist as physically discrete units suitable as a unitary dosage for animals, especially mammals, and most especially humans, wherein each unit will contain a predetermined quantity of active material calculated to produce the desired immunogenic effect in association with the required diluent. The dose of said vaccine or inoculum according to the present invention is administered at least once. In order to increase the antibody level, a second or booster dose may be administered at some time after the initial dose. The need for, and timing of, such booster dose will, of course, be determined within the sound judgment of the administrator of such vaccine or inoculum and according to sound principles well known in the art. For example, such booster dose could reasonably be expected to be advantageous at some time between about 2 weeks to about 6 months following the initial vaccination. Subsequent doses may be administered as indicated.

The nucleic acid sequences, viruses and polypeptides of the present invention can also be administered for purposes of therapy, where a mammal, especially a primate, and most especially a human, is already infected, as shown by well known diagnostic measures. When the nucleic acid sequences, viruses or polypeptides of the present invention are used for such

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therapeutic purposes, much of the same criteria will apply
as when it is used as a vaccine, except that inoculation
will occur post-infection. Thus, when the nucleic acid
sequences, viruses or polypeptides of the present
5 invention are used as therapeutic agents in the treatment
of infection, the therapeutic agent comprises a
pharmaceutical composition containing a sufficient amount
of said nucleic acid sequences, viruses or polypeptides so
10 as to elicit a therapeutically effective response in the
organism to be treated. Of course, the amount of
pharmaceutical composition to be administered will, as for
vaccines, vary depending on the immunogen contained
therein (nucleic acid, polypeptide, virus) and on the
15 route of administration.

The therapeutic agent according to the present
invention can thus be administered by, subcutaneous,
intramuscular or intradermal routes. One skilled in the
20 art will certainly appreciate that the amounts to be
administered for any particular treatment protocol can be
readily determined without undue experimentation. Of
course, the actual amounts will vary depending on the
route of administration as well as the sex, age, and
25 clinical status of the subject which, in the case of human
patients, is to be determined with the sound judgment of
the clinician.

The therapeutic agent of the present invention
30 can be employed in such forms as capsules, liquid
solutions, suspensions or elixirs, or sterile liquid forms
such as solutions or suspensions. Any inert carrier is
preferably used, such as saline, phosphate-buffered

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saline, or any such carrier in which the HCV of the present invention can be suitably suspended. The therapeutic agents may be in the form of single dose preparations or in the multi-dose flasks which can be
5 utilized for mass-treatment programs of both animals and humans. Of course, when the nucleic acid sequences, viruses or polypeptides of the present invention are used as therapeutic agents they may be administered as a single
10 dose or as a series of doses, depending on the situation as determined by the person conducting the treatment.

The nucleic acids, polypeptides and viruses of the present invention can also be utilized in the production of antibodies against HCV. The term "antibody"
15 is herein used to refer to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules. Examples of antibody molecules are intact immunoglobulin molecules, substantially intact
20 immunoglobulin molecules and portions of an immunoglobulin molecule, including those portions known in the art as Fab, F(ab')₂ and F(v) as well as chimeric antibody molecules.

25 Thus, the polypeptides, viruses and nucleic acid sequences of the present invention can be used in the generation of antibodies that immunoreact (i.e., specific binding between an antigenic determinant-containing molecule and a molecule containing an antibody combining
30 site such as a whole antibody molecule or an active portion thereof) with antigenic determinants on the surface of hepatitis C virus particles.

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The present invention therefore also relates to antibodies produced following immunization with the nucleic acid sequences, viruses or polypeptides of the present invention. These antibodies are typically produced by immunizing a mammal with an immunogen or vaccine to induce antibody molecules having immunospecificity for polypeptides or viruses produced in response to infection with the nucleic acid sequences of the present invention. When used in generating such antibodies, the nucleic acid sequences, viruses, or polypeptides of the present invention may be linked to some type of carrier molecule. The resulting antibody molecules are then collected from said mammal. Antibodies produced according to the present invention have the unique advantage of being generated in response to authentic, functional polypeptides produced according to the actual cloned HCV genome.

The antibody molecules of the present invention may be polyclonal or monoclonal. Monoclonal antibodies are readily produced by methods well known in the art. Portions of immunoglobulin molecules, such as Fabs, as well as chimeric antibodies, may also be produced by methods well known to those of ordinary skill in the art of generating such antibodies.

The antibodies according to the present invention may also be contained in blood plasma, serum, hybridoma supernatants, and the like. Alternatively, the antibody of the present invention is isolated to the extent desired by well known techniques such as, for example, using DEAE Sephadex. The antibodies produced

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according to the present invention may be further purified
so as to obtain specific classes or subclasses of antibody
such as IgM, IgG, IgA, and the like. Antibodies of the
IgG class are preferred for purposes of passive
5 protection.

The antibodies of the present invention are
useful in the prevention and treatment of diseases caused
by hepatitis C virus in animals, especially mammals, and
10 most especially humans.

In providing the antibodies of the present
invention to a recipient mammal, preferably a human, the
dosage of administered antibodies will vary depending on
such factors as the mammal's age, weight, height, sex,
15 general medical condition, previous medical history, and
the like.

In general, it will be advantageous to provide
the recipient mammal with a dosage of antibodies in the
20 range of from about 1 mg/kg body weight to about 10 mg/kg
body weight of the mammal, although a lower or higher dose
may be administered if found desirable. Such antibodies
will normally be administered by intravenous or
intramuscular route as an inoculum. The antibodies of the
25 present invention are intended to be provided to the
recipient subject in an amount sufficient to prevent,
lessen or attenuate the severity, extent or duration of
any existing infection.

30 The antibodies prepared by use of the nucleic
acid sequences, viruses or polypeptides of the present
invention are also highly useful for diagnostic purposes.
For example, the antibodies can be used as in vitro

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diagnostic agents to test for the presence of HCV in
biological samples taken from animals, especially humans.
Such assays include, but are not limited to,
radioimmunoassays, EIA, fluorescence, Western blot
5 analysis and ELISAs. In one such embodiment, the
biological sample is contacted with antibodies of the
present invention and a labeled second antibody is used to
detect the presence of HCV to which the antibodies are
10 bound.

Such assays may be, for example, a direct
protocol (where the labeled first antibody is
immunoreactive with the antigen, such as, for example, a
polypeptide on the surface of the virus), an indirect
15 protocol (where a labeled second antibody is reactive with
the first antibody), a competitive protocol (such as would
involve the addition of a labeled antigen), or a sandwich
protocol (where both labeled and unlabeled antibody are
20 used), as well as other protocols well known and described
in the art.

In one embodiment, an immunoassay method would
utilize an antibody specific for HCV envelope determinants
and would further comprise the steps of contacting a
25 biological sample with the HCV-specific antibody and then
detecting the presence of HCV material in the test sample
using one of the types of assay protocols as described
above. Polypeptides and antibodies produced according to
30 the present invention may also be supplied in the form of
a kit, either present in vials as purified material, or
present in compositions and suspended in suitable diluents
as previously described.

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In a preferred embodiment, such a diagnostic test kit for detection of HCV antigens in a test sample comprises in combination a series of containers, each container a reagent needed for such assay. Thus, one such container would contain a specific amount of HCV-specific antibody as already described, a second container would contain a diluent for suspension of the sample to be tested, a third container would contain a positive control and an additional container would contain a negative control. An additional container could contain a blank.

For all prophylactic, therapeutic and diagnostic uses, the antibodies of the invention and other reagents, plus appropriate devices and accessories, may be provided in the form of a kit so as to facilitate ready availability and ease of use.

The present invention also relates to the use of nucleic acid sequences and polypeptides of the present invention to screen potential antiviral agents for antiviral activity against HCV. Such screening methods are known by those of skill in the art. Generally, the antiviral agents are tested at a variety of concentrations, for their effect on preventing viral replication in cell culture systems which support viral replication, and then for an inhibition of infectivity or of viral pathogenicity (and a low level of toxicity) in an animal model system.

In one embodiment, animal cells (especially human cells) transfected with the nucleic acid sequences of the invention are cultured in vitro and the cells are treated with a candidate antiviral agent (a chemical,

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peptide etc.) for antiviral activity by adding the candidate agent to the medium. The treated cells are then exposed, possibly under transfecting or fusing conditions known in the art, to the nucleic acid sequences of the present invention. A sufficient period of time would then be allowed to pass for infection to occur, following which the presence or absence of viral replication would be determined versus untreated control cells by methods known to those of ordinary skill in the art. Such methods include, but are not limited to, the detection of viral antigens in the cell, for example, by immunofluorescent procedures well known in the art; the detection of viral polypeptides by Western blotting using antibodies specific therefor; the detection of newly transcribed viral RNA within the cells by RT-PCR; and the detection of the presence of live, infectious virus particles by injection of cell culture medium or cell lysates into healthy, susceptible animals, with subsequent exhibition of the symptoms of HCV infection. A comparison of results obtained for control cells (treated only with nucleic acid sequence) with those obtained for treated cells (nucleic acid sequence and antiviral agent) would indicate, the degree, if any, of antiviral activity of the candidate antiviral agent. Of course, one of ordinary skill in the art would readily understand that such cells can be treated with the candidate antiviral agent either before or after exposure to the nucleic acid sequence of the present invention so as to determine what stage, or stages, of viral infection and replication said agent is effective against.

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In an alternative embodiment, a protease such as NS3 protease produced from a nucleic acid sequence of the invention may be used to screen for protease inhibitors which may act as antiviral agents. The structural and nonstructural regions of the HCV genome, including nucleotide and amino acid locations, have been determined, for example, as depicted in Houghton, M. (1996), Fig. 1; and Major, M.E. et al. (1997), Table 1.

Such above-mentioned protease inhibitors may take the form of chemical compounds or peptides which mimic the known cleavage sites of the protease and may be screened using methods known to those of skill in the art (Houghton, M. (1996) and Major, M.E. et al. (1997)). For example, a substrate may be employed which mimics the protease's natural substrate, but which provides a detectable signal (e.g. by fluorimetric or colorimetric methods) when cleaved. This substrate is then incubated with the protease and the candidate protease inhibitor under conditions of suitable pH, temperature etc. to detect protease activity. The proteolytic activities of the protease in the presence or absence of the candidate inhibitor are then determined.

In yet another embodiment, a candidate antiviral agent (such as a protease inhibitor) may be directly assayed in vivo for antiviral activity by administering the candidate antiviral agent to a chimpanzee transfected with a nucleic acid sequence of the invention and then measuring viral replication in vivo via methods such as RT-PCR. Of course, the chimpanzee may be treated with the candidate agent either before or after transfection with

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the infectious nucleic acid sequence so as to determine what stage, or stages, of viral infection and replication the agent is effective against.

5 The invention also provides that the nucleic acid sequences, viruses and polypeptides of the invention may be supplied in the form of a kit, alone or in the form of a pharmaceutical composition.

10 All scientific publication and/or patents cited herein are specifically incorporated by reference. The following examples illustrate various aspects of the invention but are in no way intended to limit the scope thereof.

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EXAMPLES

MATERIALS AND METHODS **For Examples 1-4**

Collection of Virus

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Hepatitis C virus was collected and used as a source for the RNA used in generating the cDNA clones according to the present invention. Plasma containing strain H77 of HCV was obtained from a patient in the acute phase of transfusion-associated non-A, non-B hepatitis 25 (Feinstone et al (1981)). Strain H77 belongs to genotype 1a of HCV (Ogata et al (1991), Inchauspe et al (1991)). The consensus sequence for most of its genome has been determined (Kolyakov et al (1996), Ogata et al (1991), 30 Inchauspe et al (1991) and Farci et al (1996)).

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RNA Purification

Viral RNA was collected and purified by conventional means. In general, total RNA from 10 μ l of H77 plasma was extracted with the TRIzol system (GIBCO BRL). The RNA pellet was resuspended in 100 μ l of 10 mM dithiothreitol (DTT) with 5% (vol/vol) RNasin (20 - 40 units/ μ l) (available from Promega) and 10 μ l aliquots were stored at -80°C. In subsequent experiments RT-PCR was performed on RNA equivalent to 1 μ l of H77 plasma, which contained an estimated 10^5 genome equivalents (GE) of HCV (Yanagi et al (1996)).

Primers used in the RT-PCR process were deduced from the genomic sequences of strain H77 according to procedures already known in the art (see above) or else were determined specifically for use herein. The primers generated for this purpose are listed in Table 1.

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Table 1. Oligonucleotides used for PCR amplification of strain H77 of HCV

Designation	Sequence (5' → 3') *
H9261F	GGCTACAGCGGGGGGAGACATTTATCACAGC
H3' X58R	TCATGCGGCTCACGGACCTTTCACAGCTAG
H9282F	GTCCAAGCTT ATCACAGCGTGTCTCATGCCCCGGCCCCG
H3' X45R	CGTCTCTAG AGGACCTTTCACAGCTAGCCGTGACTAGGG
H9375F	TGAAGGTTGGGGTAAACACTCCGGCCTCTTAGGCCATT
H3' X-35R	ACATGATCTGCAGAGAGGCCAGTATCAGCACTCTC
H9386F	GTCCAAGCTTACGCGT AAACACTCCGGCCTC CTTAAGCCATTC CTG
H3' X-38R	CGTCTCTAG ACATGATCTGCAGAGAGGCCAGTATCAGCACTCTCTGC
H1	TTTTTTTTGCGGCCGCTAATACGACTCACTATAGCCAGCCCCCTGAT -
	GGGGGCGACACTCCACCATG
A1	ACTGTCTTCACGCAGAAAGCGTCTAGCCAT
H9417R	CGTCTCTAG ACAGGAAATGG CTTAAGAGGCCGAGTGT TTACC
* HCV sequences are shown in plain text, non-HCV-specific sequences are shown in boldface and artificial cleavage sites used for cDNA cloning are underlined. The core sequence of the T7 promoter in primer H1 is shown in italics.	
Primers for long RT-PCR were size-purified.	

cDNA Synthesis

The RNA was denatured at 65°C for 2 min, and cDNA synthesis was performed in a 20 µl reaction volume with Superscript II reverse transcriptase (from GIBCO/BRL) at 42 °C for 1 hour using specific antisense primers as described previously (Tellier et al (1996)). The cDNA mixture was treated with RNase H and RNase T1 (GIBCO/BRL) for 20 min at 37 °C.

Amplification and Cloning of the 3' UTR

The 3' UTR of strain H77 was amplified by PCR in two different assays. In both of these nested PCR reactions the first round of PCR was performed in a total volume of 50 µl in 1 x buffer, 250 µmol of each deoxynucleoside triphosphate (dNTP; Pharmacia), 20 pmol

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each of external sense and antisense primers, 1 μ l of the Advantage KlenTaq polymerase mix (from Clontech) and 2 μ l of the final cDNA reaction mixture. In the second round of PCR, 5 μ l of the first round PCR mixture was added to 45 μ l of PCR mixture prepared as already described. Each round of PCR (35 cycles), which was performed in a Perkin Elmer DNA thermal cycler 480, consisted of denaturation at 94 °C for 1 min (in 1st cycle 1 min 30 sec), annealing at 60°C for 1 min and elongation at 68°C for 2 min. In one experiment a region from NS5B to the conserved region of the 3' UTR was amplified with the external primers H9261F and H3'X58R, and the internal primers H9282F and H3'X45R (Table 1). In another experiment, a segment of the variable region to the very end of the 3' UTR was amplified with the external primers H9375F and H3'X-35R, and the internal primers H9386F and H3'X-38R (Table 1, Fig. 1). Amplified products were purified with QIAquick PCR purification kit (from QIAGEN), digested with *Hind* III and *Xba* I (from Promega), purified by either gel electrophoresis or phenol/chloroform extraction, and then cloned into the multiple cloning site of plasmid pGEM-9zf(-) (Promega) or pUC19 (Pharmacia). Cloning of cDNA into the vector was performed with T4 DNA ligase (Promega) by standard procedures.

Amplification of Near Full-Length H77 Genomes by Long PCR

The reactions were performed in a total volume of 50 μ l in 1 x buffer, 250 μ mol of each dNTP, 10 pmol each of sense and antisense primers, 1 μ l of the Advantage

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KlenTaq polymerase mix and 2 μ l of the cDNA reaction
mixture (Tellier et al (1996)). A single PCR round of 35
cycles was performed in a Robocycler thermal cycler (from
Stratagene), and consisted of denaturation at 99 °C for 35
5 sec, annealing at 67 °C for 30 sec and elongation at 68 °C
for 10 min during the first 5 cycles, 11 min during the
next 10 cycles, 12 min during the following 10 cycles and
13 min during the last 10 cycles. To amplify the complete
10 ORF of HCV by long RT-PCR we used the sense primers H1 or
A1 deduced from the 5' UTR and the antisense primer H9417R
deduced from the variable region of the 3' UTR (Table 1,
Fig. 1).

15 Construction of Full-Length H77 cDNA Clones

The long PCR products amplified with H1 and
H9417R primers were cloned directly into pGEM-9zf(-) after
20 digestion with Not I and Xba I (from Promega) (as per
Fig. 1). Two clones were obtained with inserts of the
expected size, pH21_r and pH50_r. Next, the chosen 3' UTR
was cloned into both pH21_r and pH50_r after digestion with
25 Afl II and Xba I (New England Biolabs). DH5 α competent
cells (GIBCO/BRL) were transformed and selected with LB
agar plates containing 100 μ g/ml ampicillin (from SIGMA).
Then the selected colonies were cultured in LB liquid
containing ampicillin at 30°C for ~18-20 hrs
30 (transformants containing full-length or near full-length
cDNA of H77 produced a very low yield of plasmid when
cultured at 37 °C or for more than 24 hrs). After small
scale preparation (Wizard Plus Minipreps DNA Purification
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Systems, Promega) each plasmid was retransformed to select a single clone, and large scale preparation of plasmid DNA was performed with a QIAGEN plasmid Maxi kit.

5 Cloning of Long RT-PCR Products Into a Cassette Vector

To improve the efficiency of cloning, a vector with consensus 5' and 3' termini of HCV strain H77 was constructed (Fig. 1). This cassette vector (pCV) was
10 obtained by cutting out the *Bam*HI fragment (nts 1358 - 7530 of the H77 genome) from pH50, followed by religation. Next, the long PCR products of H77 amplified with H1 and H9417R or A1 and H9417R primers were purified (Geneclean
15 spin kit; BIO 101) and cloned into pCV after digestion with *Age* I and *Afl* II (New England Biolabs) or with *P*in AI (isoschizomer of *Age* I) and *B*fr I (isoschizomer of *Afl* II) (Boehringer Mannheim). Large scale preparations of the
20 plasmids containing full-length cDNA of H77 were performed as described above.

Construction of H77 Consensus Chimeric cDNA Clone

A full-length cDNA clone of H77 with an ORF
25 encoding the consensus amino acid sequence was constructed by making a chimera from four of the cDNA clones obtained above. This consensus chimera, pCV-H77C, was constructed
in two ligation steps by using standard molecular
30 procedures and convenient cleavage sites and involved first a two piece ligation and then a three piece ligation. Large scale preparation of pCV-H77C was performed as already described.

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In Vitro Transcription

Plasmids containing the full-length HCV cDNA were linearized with Xba I (from Promega), and purified by phenol/chloroform extraction and ethanol precipitation. A
5 100 μ l reaction mixture containing 10 μ g of linearized plasmid DNA, 1 x transcription buffer, 1 mM ATP, CTP, GTP and UTP, 10mM DTT , 4% (v/v) RNasin (20-40 units/ μ l) and 2
10 μ l of T7 RNA polymerase (Promega) was incubated at 37 °C for 2 hrs. Five μ l of the reaction mixture was analyzed by agarose gel electrophoresis followed by ethidium bromide staining. The transcription reaction mixture was
15 diluted with 400 μ l of ice-cold phosphate-buffered saline without calcium or magnesium, immediately frozen on dry ice and stored at -80 °C. The final nucleic acid mixture was injected into chimpanzees within 24 hrs.

Intrahepatic Transfection of Chimpanzees

20 Laparotomy was performed and aliquots from two transcription reactions were injected into 6 sites of the exposed liver (Emerson et al (1992)). Serum samples were
25 collected weekly from chimpanzees and monitored for liver enzyme levels and anti-HCV antibodies. Weekly samples of 100 μ l of serum were tested for HCV RNA in a highly sensitive nested RT-PCR assay with AmpliTaq Gold (Perkin
30 Elmer) (Yanagi et al (1996); Bukh et al (1992)). The genome titer of HCV was estimated by testing 10-fold serial dilutions of the extracted RNA in the RT-PCR assay (Yanagi et al (1996)). The two chimpanzees used in this

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study were maintained under conditions that met all requirements for their use in an approved facility.

The consensus sequence of the complete ORF from HCV genomes recovered at week 2 post inoculation (p.i) was determined by direct sequencing of PCR products obtained in long RT-PCR with primers A1 and H9417R followed by nested PCR of 10 overlapping fragments. The consensus sequence of the variable region of the 3' UTR was determined by direct sequencing of an amplicon obtained in nested RT-PCR as described above. Finally, we amplified selected regions independently by nested RT-PCR with AmpliTaq Gold.

15 Sequence Analysis

Both strands of DNA from PCR products, as well as plasmids, were sequenced with the ABI PRISM Dye Termination Cycle Sequencing Ready Reaction Kit using Taq DNA polymerase (Perkin Elmer) and about 100 specific sense and antisense sequence primers.

The consensus sequence of HCV strain H77 was determined in two different ways. In one approach, overlapping PCR products were directly sequenced, and amplified in nested RT-PCR from the H77 plasma sample. The sequence analyzed (nucleotides (nts) 35-9417) included the entire genome except the very 5' and 3' termini. In the second approach, the consensus sequence of nts 157-9384 was deduced from the sequences of 18 full-length cDNA clones.

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EXAMPLE 1

Variability in the sequence of the 3' UTR of HCV strain H77

5 The heterogeneity of the 3' UTR was analyzed by
cloning and sequencing of DNA amplicons obtained in nested
RT-PCR. 19 clones containing sequences of the entire
variable region, the poly U-UC region and the adjacent 19
10 nt of the conserved region, and 65 clones containing
sequences of the entire poly U-UC region and the first 63
nts of the conserved region were analyzed. This analysis
confirmed that the variable region consisted of 43 nts,
including two conserved termination codons (Han et al
15 (1992)). The sequence of the variable region was highly
conserved within H77 since only 3 point mutations were
found among the 19 clones analyzed. A poly U-UC region
was present in all 84 clones analyzed. However, its
20 length varied from 71-141 nts. The length of the poly U
region was 9-103 nts, and that of the poly UC region was
35-85 nts. The number of C residues increased towards the
3' end of the poly UC region but the sequence of this
25 region is not conserved. The first 63 nts of the
conserved region were highly conserved among the clones
analyzed, with a total of only 14 point mutations. To
confirm the validity of the analysis, the 3' UTR was
reamplified directly from a full-length cDNA clone of HCV
30 (see below) by the nested-PCR procedure with the primers
in the variable region and at the very 3' end of the HCV
genome and cloned the PCR product. Eight clones had 1-7
nt deletions in the poly U region. Furthermore, although

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the C residues of the poly UC region were maintained, the spacing of these varied because of 1-2 nt deletions of U residues. These deletions must be artifacts introduced by PCR and such mistakes may have contributed to the heterogeneity originally observed in this region.

However, the conserved region of the 3' UTR was amplified correctly, suggesting that the deletions were due to difficulties in transcribing a highly repetitive sequence.

One of the 3' UTR clones was selected for engineering of full-length cDNA clones of H77. This clone had the consensus variable sequence except for a single point mutation introduced to create an Afl II cleavage site, a poly U-UC stretch of 81 nts with the most commonly observed UC pattern and the consensus sequence of the complete conserved region of 101 nts, including the distal 38 nts which originated from the antisense primer used in the amplification. After linearization with Xba I, the DNA template of this clone had the authentic 3' end.

EXAMPLE 2

The Entire Open Reading Frame of H77 Amplified in One Round of Long RT-PCR

It had been previously demonstrated that a 9.25 kb fragment of the HCV genome from the 5' UTR to the 3' end of NS5B could be amplified from 10^4 GE (genome equivalents) of H77 by a single round of long RT-PCR (Tellier et al (1996a)). In the current study, by optimizing primers and cycling conditions, the entire ORF of H77 was amplified in a single round of long RT-PCR with primers from the 5' UTR and the variable region of the 3'

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UTR. In fact, 9.4 kb of the H77 genome (H product: from the very 5' end to the variable region of the 3' UTR) could be amplified from 10^5 GE or 9.3 kb (A product: from within the 5' UTR to the variable region of the 3' UTR) from 10^4 GE or 10^5 GE, in a single round of long RT-PCR (Fig. 2). The PCR products amplified from 10^5 GE of H77 were used for engineering full-length cDNA clones (see below).

EXAMPLE 3

Construction of Multiple Full-Length
cDNA Clones of H77 in a Single Step by
Cloning of Long RT-PCR Amplicons Directly
into a Cassette Vector with Fixed 5' and 3' Termini

Direct cloning of the long PCR products (H), which contained a 5' T7 promoter, the authentic 5' end, the entire ORF of H77 and a short region of the 3' UTR, into pGEM-9zf(-) vector by Not I and Xba I digestion was first attempted. However, among the 70 clones examined all but two had inserts that were shorter than predicted. Sequence analysis identified a second Not I site in the majority of clones, which resulted in deletion of the nts past position 9221. Only two clones (pH21_r and pH50_r) were missing the second Not I site and had the expected 5' and 3' sequences of the PCR product. Therefore, full-length cDNA clones (pH21 and pH50) were constructed by inserting the chosen 3' UTR into pH21_r and pH50_r, respectively. Sequence analysis revealed that clone pH21 had a complete full-length sequence of H77; this clone was tested for infectivity. The second clone, pH50, had one nt deletion

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in the ORF at position 6365; this clone was used to make a cassette vector.

The complete ORF was amplified by constructing a cassette vector with fixed 5' and 3' termini as an intermediate of the full-length cDNA clones. This vector (pCV) was constructed by digestion of clone pH50 with *Bam*HI, followed by religation, to give a shortened plasmid readily distinguished from plasmids containing the full-length insert. Attempts to clone long RT-PCR products (H) into pCV by *Age* I and *Afl* II yielded only 1 of 23 clones with an insert of the expected size. In order to increase the efficiency of cloning, we repeated the procedure but used *Pin* A I and *Bfr* I instead of the respective isoschizomers *Age* I and *Afl* II. By this protocol, 24 of 31 H clones and 30 of 35 A clones had the full-length cDNA of H77 as evaluated by restriction enzyme digestion. A total of 16 clones, selected at random, were each retransformed, and individual plasmids were purified and completely sequenced.

EXAMPLE 4

Demonstration of Infectious Nature of Transcripts of a cDNA Clone Representing the Consensus Sequence of Strain H77

A consensus chimera was constructed from 4 of the full-length cDNA clones with just 2 ligation steps. The final construct, pCV-H77C, had 11 nt differences from the consensus sequence of H77 in the ORF (Fig. 3). However, 10 of these nucleotide differences represented silent mutations. The chimeric clone differed from the

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consensus sequence at only one amino acid [L instead of F at position 790]. Among the 18 ORFs analyzed above, the F residue was found in 11 clones and the L residue in 7 clones. However, the L residue was dominant in other isolates of genotype 1a, including a first passage of H77 in a chimpanzee (Inchauspe et al (1991)).

To test the infectivity of the consensus chimeric clone of H77 intrahepatic transfection of a chimpanzee was performed. The pCV-H77C clone was linearized with Xba I and transcribed *in vitro* by T7 RNA polymerase (Fig. 2). The transcription mixture was next injected into 6 sites of the liver of chimpanzee 1530. The chimpanzee became infected with HCV as measured by detection of 10^2 GE/ml of viral genome at week 1 p.i. Furthermore, the HCV titer increased to 10^4 GE/ml at week 2 p.i., and reached 10^6 GE/ml by week 8 p.i. The viremic pattern observed in the early phase of the infection with the recombinant virus was similar to that observed in chimpanzees inoculated intravenously with strain H77 or other strains of HCV (Shimizu (1990)).

The sequence of the HCV genomes from the serum sample collected at week 2 p.i. was analyzed. The consensus sequence of nts 298-9375 of the recovered genomes was determined by direct sequencing of PCR products obtained in long RT-PCR followed by nested PCR of 10 overlapping fragments. The identity to clone pCV-H77C sequence was 100%. The consensus sequence of nts 96-291, 1328-1848, 3585-4106, 4763-5113 and 9322-9445 was determined from PCR products obtained in different nested RT-PCR assays. The identity of these sequences with pCV-

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H77C was also 100%. These latter regions contained 4 mutations unique to the consensus chimera, including the artificial Afl II cleavage site in the 3' UTR. Therefore, RNA transcripts of this clone of HCV were infectious.

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The infectious nature of the consensus chimera indicates that the regions of the 5' and 3' UTRs incorporated into the cassette vector do not destroy viability. This makes it highly advantageous to use the cassette vector to construct infectious cDNA clones of other HCV strains when the consensus sequence for each ORF is inserted.

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In addition, two complete full-length clones (dubbed pH21 and pCV-H11) constructed were not infectious, as shown by intrahepatic injection of chimpanzees with the corresponding RNA transcripts. Thus, injection of the transcription mixture into 3 sites of the exposed liver resulted in no observable HCV replication and weekly serum samples were negative for HCV RNA at weeks 1 - 17 p.i. in a highly sensitive nested RT-PCR assay. The cDNA template injected along with the RNA transcripts was also not detected in this assay.

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Moreover, the chimpanzee remained negative for antibodies to HCV throughout the follow-up. Subsequent sequence analysis revealed that 7 of 16 additional clones were defective for polyprotein synthesis and all clones had multiple amino acid mutations compared with the consensus sequence of the parent strain. For example, clone pH21, which was not infectious, had 7 amino acid substitutions in the entire predicted polyprotein compared with the consensus sequence of H77 (Fig. 3). The most

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notable mutation was at position 1026, which changed L to Q, altering the cleavage site between NS2 and NS3 (Reed (1995)). Clone pCV-H11, also non-infectious, had 21 amino acid substitutions in the predicted polyprotein compared with the consensus sequence of H77 (Fig. 3). The amino acid mutation at position 564 eliminated a highly conserved C residue in the E2 protein (Okamoto (1992a)).

EXAMPLE 4A

The chimpanzee of Example 4, designated 1530, was monitored out to 32 weeks p.i. for serum enzyme levels (ALT) and the presence of anti-HCV antibodies, HCV RNA, and liver histopathology. The results are shown in Figure 18B.

A second chimp, designated 1494, was also transfected with RNA transcripts of the pCV-H77C clone and monitored out to 17 weeks p.i. for the presence of anti-HCV antibodies, HCV RNA and elevated serum enzyme levels. The results are shown in Figure 18A.

MATERIALS AND METHODS for Examples 5-10

Source Of HCV Genotype 1b

An infectious plasma pool (second chimpanzee passage) containing strain HC-J4, genotype 1b, was prepared from acute phase plasma of a chimpanzee experimentally infected with serum containing HC-J4/91 (Okamoto et al., 1992b). The HC-J4/91 sample was obtained from a first chimpanzee passage during the chronic phase of hepatitis C about 8 years after experimental infection.

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The consensus sequence of the entire genome, except for the very 3' end, was determined previously for HC-J4/91 (Okamoto et al., 1992b).

5 Preparation Of HCV RNA

Viral RNA was extracted from 100 μ l aliquots of the HC-J4 plasma pool with the TRIzol system (GIBCO BRL). The RNA pellets were each resuspended in 10 μ l of 10 mM
10 dithiothreitol (DTT) with 5% (vol/vol) RNasin (20-40 units/ μ l) (Promega) and stored at -80°C or immediately used for cDNA synthesis.

15 Amplification And Cloning Of The 3' UTR

A region spanning from NS5B to the conserved region of the 3' UTR was amplified in nested RT-PCR using the procedure of Yanagi et al., (1997).

20 In brief, the RNA was denatured at 65°C for 2 minutes, and cDNA was synthesized at 42°C for 1 hour with Superscript II reverse transcriptase (GIBCO BRL) and primer H3'X58R (Table 1) in a 20 μ l reaction volume. The
25 cDNA mixture was treated with RNase H and RNase T1 (GIBCO BRL) at 37°C for 20 minutes. The first round of PCR was performed on 2 μ l of the final cDNA mixture in a total volume of 50 μ l with the Advantage cDNA polymerase mix (Clontech) and external primers H9261F (Table 1) and
30 H3'X58R (Table 1). In the second round of PCR [internal primers H9282F (Table 1) and H3'X45R (Table 1)], 5 μ l of the first round PCR mixture was added to 45 μ l of the PCR reaction mixture. Each round of PCR (35 cycles), was
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performed in a DNA thermal cycler 480 (Perkin Elmer) and consisted of denaturation at 94°C for 1 minute (1st cycle: 1 minute 30 sec), annealing at 60°C for 1 minute and elongation at 68°C for 2 minutes. After purification with QIAquick PCR purification kit (QIAGEN), digestion with *HindIII* and *XbaI* (Promega), and phenol/chloroform extraction, the amplified products were cloned into pGEM-9zf(-) (Promega) (Yanagi et al., 1997).

Amplification And Cloning Of The Entire ORF

A region from within the 5' UTR to the variable region of the 3' UTR of strain HC-J4 was amplified by long RT-PCR (Fig. 1) (Yanagi et al., 1997). The cDNA was synthesized at 42°C for 1 hour in a 20 µl reaction volume with Superscript II reverse transcriptase and primer J4-9405R (5'-GCCTATTGGCCTGGAGTGGTTAGCTC-3'), and treated with RNases as above. The cDNA mixture (2 µl) was amplified by long PCR with the Advantage cDNA polymerase mix and primers A1 (Table 1) (Bukh et al., 1992; Yanagi et al., 1997) and J4-9398R (5'-**AGGATGGCCTTAAG**GCCTGGAGTGGTTAGCTCCCCGTTCA-3'). Primer J4-9398R contained extra bases (**bold**) and an artificial *AflII* cleavage site (underlined). A single PCR round was performed in a Robocycler thermal cycler (Stratagene), and consisted of denaturation at 99°C for 35 seconds, annealing at 67°C for 30 seconds and elongation at 68°C for 10 minutes during the first 5 cycles, 11 minutes during the next 10 cycles, 12 minutes during the following 10 cycles and 13 minutes during the last 10 cycles.

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After digesting the long PCR products obtained from strain HC-J4 with *PinAI* (isoschizomer of *AgeI*) and *BfrI* (isoschizomer of *AflIII*) (Boehringer Mannheim), attempts were made to clone them directly into a cassette vector (pCV), which contained the 5' and 3' termini of strain H77 (Figure 1) but no full-length clones were obtained. Accordingly, to improve the efficiency of cloning, the PCR product was further digested with *BglII* (Boehringer Mannheim) and the two resultant genome fragments [L fragment: *PinAI/BglIII*, nts 156 - 8935; S fragment: *BglIII/BrfI*, nts 8936 - 9398] were separately cloned into pCV (Figure 6).

DH5 α competent cells (GIBCO BRL) were transformed and selected on LB agar plates containing 100 μ g/ml ampicillin (SIGMA) and amplified in LB liquid cultures at 30°C for 18-20 hours.

Sequence analysis of 9 plasmids containing the S fragment (miniprep samples) and 9 plasmids containing the L fragment (maxiprep samples) were performed as described previously (Yanagi et al., 1997). Three L fragments, each encoding a distinct polypeptide, were cloned into pCV-J4S9 (which contained an S fragment encoding the consensus amino acid sequence of HC-J4) to construct three chimeric full-length HCV cDNAs (pCV-J4L2S, pCV-J4L4S and pCV-J4L6S) (Fig. 6). Large scale preparation of each clone was performed as described previously with a QIAGEN plasmid Maxi kit (Yanagi et al., 1997) and the authenticity of each clone was confirmed by sequence analysis.

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Sequence Analysis

Both strands of DNA were sequenced with the ABI PRISM Dye Termination Cycle Sequencing Ready Reaction Kit using Taq DNA polymerase (Perkin Elmer) and about 90 specific sense and antisense primers. Analyses of genomic sequences, including multiple sequence alignments and tree analyses, were performed with GeneWorks (Oxford Molecular Group) (Bukh et al., 1995).

The consensus sequence of strain HC-J4 was determined by direct sequencing of PCR products (nts 11 - 9412) and by sequence analysis of multiple cloned L and S fragments (nts 156 - 9371). The consensus sequence of the 3' UTR (3' variable region, polypyrimidine tract and the first 16 nucleotides of the conserved region) was determined by analysis of 24 cDNA clones.

Intrahepatic Transfection Of A Chimpanzee With Transcribed RNA

Two in vitro transcription reactions were performed with each of the three full-length clones. In each reaction 10 µg of plasmid DNA linearized with Xba I (Promega) was transcribed in a 100 µl reaction volume with T7 RNA polymerase (Promega) at 37°C for 2 hours as described previously (Yanagi et al., 1997). Five µl of the final reaction mixture was analyzed by agarose gel electrophoresis and ethidium bromide staining (Fig. 5). Each transcription mixture was diluted with 400 µl of ice-cold phosphate-buffered saline without calcium or magnesium and then the two aliquots from the same cDNA

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clone were combined, immediately frozen on dry ice and stored at -80°C. Within 24 hours after freezing the transcription mixtures were injected into the chimpanzee by percutaneous intrahepatic injection that was guided by
5 ultrasound. Each inoculum was individually injected (5-6 sites) into a separate area of the liver to prevent complementation or recombination. The chimpanzee was maintained under conditions that met all requirements for
10 its use in an approved facility.

Serum samples were collected weekly from the chimpanzee and monitored for liver enzyme levels and anti-HCV antibodies. Weekly samples of 100 µl of serum were tested for HCV RNA in a sensitive nested RT-PCR assay
15 (Bukh et al., 1992, Yanagi et al., 1996) with AmpliTaq Gold DNA polymerase. The genome equivalent (GE) titer of HCV was determined by testing 10-fold serial dilutions of the extracted RNA in the RT-PCR assay (Yanagi et al.,
20 1996) with 1 GE defined as the number of HCV genomes present in the highest dilution which was positive in the RT-nested PCR assay.

To identify which of the three clones was
25 infectious in vivo, the NS3 region (nts 3659 - 4110) from the chimpanzee serum was amplified in a highly sensitive and specific nested RT-PCR assay with AmpliTaq Gold DNA polymerase and the PCR products were cloned with a TA cloning kit (Invitrogen). In addition, the consensus
30 sequence of the nearly complete genome (nts 11 - 9441) was determined by direct sequencing of overlapping PCR products.

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EXAMPLE 5Sequence Analysis Of Infectious Plasma Pool
Of Strain HC-J4 Used As The Cloning Source

5 As an infectious cDNA clone of a genotype 1a strain of HCV had been obtained only after the ORF was engineered to encode the consensus polypeptide (Kolykhalov et al., 1997; Yanagi et al., 1997), a detailed sequence
10 analysis of the cloning source was performed to determine the consensus sequence prior to constructing an infectious cDNA clone of a 1b genotype.

15 A plasma pool of strain HC-J4 was prepared from acute phase plasmapheresis units collected from a chimpanzee experimentally infected with HC-J4/91 (Okamoto et al., 1992b). This HCV pool had a PCR titer of 10^4 - 10^5 GE/ml and an infectivity titer of approximately 10^3 chimpanzee infectious doses per ml.

20 The heterogeneity of the 3' UTR of strain HC-J4 was determined by analyzing 24 clones of nested RT-PCR product. The consensus sequence was identical to that previously published for HC-J4/91 (Okamoto et al., 1992b),
25 except at position 9407 (see below). The variable region consisted of 41 nucleotides (nts. 9372 - 9412), including two in-frame termination codons. Furthermore, its sequence was highly conserved except at positions 9399 (19 A and 5 T clones) and 9407 (17 T and 7 A clones). The
30 poly U-UC region varied slightly in composition and greatly in length (31-162 nucleotides). In the conserved region, the first 16 nucleotides of 22 clones were identical to those previously published for other genotype

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1 strains, whereas two clones each had a single point mutation. These data suggested that the structural organization at the 3' end of HC-J4 was similar to that of the infectious clone of a genotype 1a strain of Yanagi et al (1997).

Next, the entire ORF of HC-J4 was amplified in a single round of long RT-PCR (Figure 5). The original plan was to clone the resulting PCR products into the PinAI and BrfI site of a HCV cassette vector (pCV), which had fixed 5' and 3' termini of genotype 1a (Yanagi et al., 1997) but since full-length clones were not obtained, two genome fragments (L and S) derived from the long RT-PCR products (Figure 6) were separately subcloned into pCV.

To determine the consensus sequence of the ORF, the sequence of 9 clones each of the L fragment (pCV-J4L) and of the S fragment (pCV-J4S) was determined and quasispecies were found at 275 nucleotide (3.05 %) and 78 amino acid (2.59 %) positions, scattered throughout the 9030 nts (3010 aa) of the ORF (Figure 7). Of the 161 nucleotide substitutions unique to a single clone, 71% were at the third position of the codon and 72 % were silent.

Each of the nine L clones represented the near complete ORF of an individual genome. The differences among the L clones were 0.30 - 1.53% at the nucleotide and 0.31 - 1.47% at the amino acid level (Figure 8). Two clones, L1 and L7, had a defective ORF due to a single nucleotide deletion and a single nucleotide insertion, respectively. Even though the HC-J4 plasma pool was obtained in the early acute phase, it appeared to contain

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at least three viral species (Figure 9). Species A contained the L1, L2, L6, L8 and L9 clones, species B the L3, L7 and L10 clones and species C the L4 clone. Although each species A clone was unique all A clones differed from all B clones at the same 20 amino acid sites and at these positions, species C had the species A sequence at 14 positions and the species B sequence at 6 positions (Figure 7).

Okamoto and coworkers (Okamoto et al., 1992b) previously determined the nearly complete genome consensus sequence of strain HC-J4 in acute phase serum of the first chimpanzee passage (HC-J4/83) as well as in chronic phase serum collected 8.2 years later (HC-J4/91). In addition, they determined the sequence of amino acids 379 to 413 (including HVR1) and amino acids 468 to 486 (including HVR2) of multiple individual clones (Okamoto et al., 1992b).

It was found by the present inventors that the sequences of individual genomes in the plasma pool collected from a chimpanzee inoculated with HC-J4/91 were all more closely related to HC-J4/91 than to HC-J4/83 (Figures 8, 9) and contained HVR amino acid sequences closely related to three of the four viral species previously found in HC-J4/91 (Figure 10).

Thus, the data presented herein demonstrate the occurrence of the simultaneous transmission of multiple species to a single chimpanzee and clearly illustrates the difficulties in accurately determining the evolution of HCV over time since multiple species with significant changes throughout the HCV genome can be present from the

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onset of the infection. Accordingly, infection of chimpanzees with monoclonal viruses derived from the infectious clones described herein will make it possible to perform more detailed studies of the evolution of HCV in vivo and its importance for viral persistence and pathogenesis.

EXAMPLE 6

Determination Of The Consensus Sequence Of HC-J4 In The Plasma Pool

The consensus sequence of nucleotides 156-9371 of HC-J4 was determined by two approaches. In one approach, the consensus sequence was deduced from 9 clones of the long RT-PCR product. In the other approach the long RT-PCR product was reamplified by PCR as overlapping fragments which were sequenced directly. The two "consensus" sequences differed at 31 (0.34%) of 9216 nucleotide positions and at 11 (0.37%) of 3010 deduced amino acid positions (Figure 7). At all of these positions a major quasispecies of strain HC-J4 was found in the plasma pool. At 9 additional amino acid positions the cloned sequences displayed heterogeneity and the direct sequence was ambiguous (Figure 7). Finally, it should be noted that there were multiple amino acid positions at which the consensus sequence obtained by direct sequencing was identical to that obtained by cloning and sequencing even though a major quasispecies was detected (Figure 7).

For positions at which the two "consensus" sequences of HC-J4 differed, both amino acids were

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included in a composite consensus sequence (Figure 7).
However, even with this allowance, none of the 9 L clones
analyzed (aa 1 - 2864) had the composite consensus
sequence: two clones did not encode the complete
5 polypeptide and the remaining 7 clones differed from the
consensus sequence by 3 - 13 amino acids (Figure 7).

EXAMPLE 7

10 Construction Of Chimeric Full-Length cDNA Clones Containing The Entire ORF Of HC-J4

The cassette vector used to clone strain H77 was
used to construct an infectious cDNA clone containing the
15 ORF of a second subtype.

15 In brief, three full-length cDNA clones were
constructed by cloning different L fragments into the
PinAI/BglIII site of pCV-J4S9, the cassette vector for
genotype 1a (Figure 6), which also contained an S fragment
20 encoding the consensus amino acid sequence of HC-J4.
Therefore, although the ORF was from strain HC-J4, most of
the 5' and 3' terminal sequences originated from strain
H77. As a result, the 5' and 3' UTR were chimeras of
25 genotypes 1a and 1b (Figure 11).

The first 155 nucleotides of the 5' UTR were
from strain H77 (genotype 1a), and differed from the
authentic sequence of HC-J4 (genotype 1b) at nucleotides
11, 12, 13, 34 and 35. In two clones (pCV-J4L2S, pCV-
30 J4L6S) the rest of the 5' UTR had the consensus sequence
of HC-J4, whereas the third clone (pCV-J4L4S) had a single
nucleotide insertion at position 207. In all 3 clones the
first 27 nucleotides of the 3' variable region of the 3'
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UTR were identical with the consensus sequence of HC-J4. The remaining 15 nucleotides of the variable region, the poly U-UC region and the 3' conserved region of the 3' UTR had the same sequence as an infectious clone of strain H77 (Figure 11).

None of the three full-length clones of HC-J4 had the ORF composite consensus sequence (Figures 7, 12). The pCV-J4L6S clone had only three amino acid changes: Q for R at position 231 (E1), V for A at position 937 (NS2) and T for S at position 1215 (NS3). The pCV-J4L4S clone had 7 amino acid changes, including a change at position 450 (E2) that eliminated a highly conserved N-linked glycosylation site (Okamoto et al., 1992a). Finally, the pCV-J4L2S clone had 9 amino acid changes compared with the consensus sequence of HC-J4. A change at position 304 (E1) mutated a highly conserved cysteine residue (Bukh et al., 1993; Okamoto et al., 1992a).

EXAMPLE 8

Transfection Of A Chimpanzee By In Vitro Transcripts Of A Chimeric cDNA

The infectivity of the three chimeric HCV clones was determined by ultra-sound-guided percutaneous intrahepatic injection into the liver of a chimpanzee of the same amount of cDNA and transcription mixture for each of the clones (Figure 5). This procedure is a less invasive procedure than the laparotomy procedure utilized by Kolykhalov et al. (1997) and Yanagi et al. (1997) and should facilitate in vivo studies of cDNA clones of HCV in

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chimpanzees since percutaneous procedures, unlike laparotomy, can be performed repeatedly.

As shown in Figure 13, the chimpanzee became infected with HCV as measured by increasing titers of 10^2 GE/ml at week 1 p.i., 10^3 GE/ml at week 2 p.i. and 10^4 - 10^5 GE/ml during weeks 3 to 10 p.i.

The viremic pattern found in the early phase of the infection was similar to that observed for the recombinant H77 virus in chimpanzees (Bukh et al., unpublished data; Kolykhalov et al., 1997; Yanagi et al., 1997). The chimpanzee transfected in the present study was chronically infected with hepatitis G virus (HGV/GBV C) (Bukh et al., 1998) and had a titer of 10^6 GE/ml at the time of HCV transfection. Although HGV/GBV-C was originally believed to be a hepatitis virus, it does not cause hepatitis in chimpanzees (Bukh et al., 1998) and may not replicate in the liver (Laskus et al., 1997). The present study demonstrated that an ongoing infection of HGV/GBV-C did not prevent acute HCV infection in the chimpanzee model.

However, to identify which of the three full-length HC-J4 clones were infectious, the NS3 region (nts. 3659 - 4110) of HCV genomes amplified by RT-PCR from serum samples taken from the infected chimpanzee during weeks 2 and 4 post-infection (p.i.) were cloned and sequenced. As the PCR primers were a complete match with each of the original three clones, this assay should not have preferentially amplified one virus over another. Sequence analysis of 26 and 24 clones obtained at weeks 2 and 4

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p.i., respectively, demonstrated that all originated from the transcripts of pCV-J4L6S.

Moreover, the consensus sequence of PCR products of the nearly complete genome (nts. 11-9441), amplified
5 from serum obtained during week 2 p.i., was identical to the sequence of pCV-J4L6S and there was no evidence of quasispecies. Thus, RNA transcripts of pCV-J4L6S, but not of pCV-J4L2S or pCV-J4L4S, were infectious in vivo. The
10 data in Figure 13 is therefore the product of the transfection of RNA transcripts of pCV-J4L6S.

In addition, the chimeric sequences of genotypes 1a and 1b in the UTRs were maintained in the infected chimpanzee. The consensus sequence of nucleotides 11 -
15 341 of the 5' UTR and the variable region of the 3' UTR, amplified from serum obtained during weeks 2 and 4 p.i., had the expected chimeric sequence of genotypes 1a and 1b (Fig. 11). Also three of four clones of the 3' UTR
20 obtained at week 2 p.i. had the chimeric sequence of the variable region, whereas a single substitution was noted in the fourth clone. However, in all four clones the poly U region was longer (2-12 nts) than expected. Also, extra
25 C and G residues were observed in this region. For the most part, the number of C residues in the poly UC region was maintained in all clones although the spacing varied. As shown previously, variations in the number of U
30 residues can reflect artifacts introduced during PCR amplification (Yanagi et al., 1997). The sequence of the first 19 nucleotides of the conserved region was maintained in all four clones. Thus, with the exception of the poly U-UC region, the genomic sequences recovered
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from the infected chimpanzee were exactly those of the chimeric infectious clone pCV-J4BL6S.

The results presented in Figure 13 therefore demonstrate that HCV polypeptide sequences other than the consensus sequence can be infectious and that a chimeric genome containing portions of the H77 termini could produce an infectious virus. In addition, these results showed for the first time that it is possible to make infectious viruses containing 5' and 3' terminal sequences specific for two different subtypes of the same major genotype of HCV.

EXAMPLE 9

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Construction Of A Chimeric 1a/1b Infectious Clone

A chimeric 1a/1b infectious clone in which the structural region of the genotype 1b infectious clone is inserted into the 1a clone of Yanagi et al. (1997) is constructed by following the protocol shown in Figure 15. The resultant chimera contains nucleotides 156-2763 of the 1b clone described herein inserted into the 1a clone of Figures 4A-4F. The sequences of the primers shown in Figure 15 which are used in constructing this chimeric clone, designated pH77CV-J4, are presented below.

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1. H2751S (Cla I/Nde I)
CGT CAT CGA TCC TCA GCG GGC ATA TGC ACT GGA CAC GGA
2. H2870R
CAT GCA CCA GCT GAT ATA GCG CTT GTA ATA TG
- 5 3. H7851S
TCC GTA GAG GAA GCT TGC AGC CTG ACG CCC
4. H9173 R(P-M)
GTA CTT GCC ACA TAT AGC AGC CCT GCC TCC TCT G
- 10 5. H9140S (P-M)
CAG AGG AGG CAG GGC TGC TAT ATG TGG CAA GTA C
6. H9417R
CGT CTC TAG ACA GGA AAT GGC TTA AGA GGC CGG AGT GTT
TAC C
- 15 7. J4-2271S
TGC AAT TGG ACT CGA GGA GAG CGC TGT AAC TTG GAG
8. J4-2776R (Nde I)
CGG TCC AAG GCA TAT GCT CGT GGT GGT AAC GCC AG

20 Transcripts of the chimeric 1a/1b clone (whose
sequence is shown in Figures 16A-16F) are then produced
and transfected into chimpanzees by the methods described
in the Materials and Methods section herein and the
transfected animals are then be subjected to biochemical
25 (ALT levels), histopathological and PCR analyses to
determine the infectivity of the chimeric clone.

EXAMPLE 10

Construction of 3' Deletion Mutants Of The 1a Infectious Clone pCV-H77C

30 Seven constructs having various deletions in the
3' untranslated region (UTR) of the 1a infectious clone
pCV-H77C were constructed as described in Figures 17A-17B.

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The 3' untranslated sequence remaining in each of the seven constructs following their respective deletions is shown in Figures 17A-17B.

5 Construct pCV-H77C(-98X) containing a deletion of the 3'-most 98 nucleotide sequences in the 3'-UTR was transcribed in vitro according to the methods described herein and 1 ml of the diluted transcription mixture was percutaneously transfected into the liver of a chimpanzee
10 with the aid of ultrasound. After three weeks, the transfection was repeated. The chimpanzee was observed to be negative for hepatitis C virus replication as measured by RT-PCR assay for 5 weeks after transfection. These
15 results demonstrate that the deleted 98 nucleotide 3'-UTR sequence was critical for production of infectious HCV and appear to contradict the reports of Dash et al. (1996) and Yoo et al. (1995) who reported that RNA transcripts from
20 cDNA clones of HCV-1 and HCV-N lacking the terminal 98 conserved nucleotides at the very 3' end of the 3'-UTR resulted in viral replication after transfection into human hematoma cell lines.

Transcripts of the (-42X) mutant (Figure 17C)
25 were also produced and transfected into a chimpanzee and transcripts of the other five deletion mutants shown in Figures 17D-17G) are to be produced and transfected into chimpanzees by the methods described herein. All
30 transfected animals are to then be assayed for viral replication via RT-PCR.

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Discussion

In two recent reports on transfection of chimpanzees, only those clones engineered to have the independently determined and slightly different consensus amino acid sequence of the polypeptide of strain H77 were infectious (Kolykhalov et al., 1997; Yanagi et al., 1997). Although the two infectious clones differed at four amino acid positions, these differences were represented in a major component of the quasispecies of the cloning source. In the present study, a single consensus sequence of strain HC-J4 could not be defined because the consensus sequence obtained by two different approaches (direct sequencing and sequencing of cloned products) differed at 20 amino acid positions, even though the same genomic PCR product was analyzed. The infectious clone differed at two positions from the composite amino acid consensus sequence, from the sequence of the 8 additional HC-J4 clones analyzed in this study and from published sequences of earlier passage samples. An additional amino acid differed from the composite consensus sequence but was found in two other HC-J4 clones analyzed in this study. The two non-infectious full-length clones of HC-J4 differed from the composite consensus sequence by only 7 and 9 amino acid differences. However, since these clones had the same termini as the infectious clone (except for a single nucleotide insertion in the 5' UTR of pCV-J4L4S), one or more of these amino acid changes in each clone was apparently deleterious for the virus.

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It was also found in the present study that HC-J4, like other strains of genotype 1b (Kolykhalov et al., 1996; Tanaka et al., 1996; Yamada et al., 1996), had a poly U-UC region followed by a terminal conserved element. The poly U-UC region appears to vary considerably so it was not clear whether changes in this region would have a significant effect on virus replication. On the other hand, the 3' 98 nucleotides of the HCV genome were previously shown to be identical among other strains of genotypes 1a and 1b (Kolykhalov et al., 1996; Tanaka et al., 1996). Thus, use of the cassette vector would not alter this region except for addition of 3 nucleotides found in strain H77 between the poly UC region and the 3' 98 conserved nucleotides.

In conclusion, an infectious clone representing a genotype 1b strain of HCV has been constructed. Thus, it has been demonstrated that it was possible to obtain an infectious clone of a second strain of HCV. In addition, it has been shown that a consensus amino acid sequence was not absolutely required for infectivity and that chimeras between the UTRs of two different genotypes could be viable.

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WHAT IS CLAIMED IS:

1. A purified and isolated nucleic acid molecule which encodes human hepatitis C virus, said molecule capable of expressing said virus when transfected into cells.
2. The nucleic acid molecule of claim 1, wherein said molecule encodes the amino acid sequence shown in Figures 14G-14H.
3. The nucleic acid molecule of claim 2, wherein said molecule comprises the nucleic acid sequence shown in Figures 14A-14F.
4. The nucleic acid molecule of claim 1, wherein said molecule encodes the amino acid sequence shown in Figures 4G-4H.
5. The nucleic acid molecule of claim 4, wherein said molecule comprises the nucleic acid sequence shown in Figures 4A-4F.
6. The nucleic acid molecule of claim 1, wherein a fragment of said molecule which encodes the structural region of hepatitis C virus has been replaced by the structural region from the genome of another hepatitis C virus strain.
7. The nucleic acid molecule of claim 6, wherein said molecule encodes the amino acid sequence shown in Figures 16G-16H.
8. The nucleic acid molecule of claim 7, wherein said molecule comprises the nucleic acid sequence shown in Figures 16A-16F.

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9. The nucleic acid molecule of claim 1,
wherein a fragment of the nucleic acid molecule which
encodes at least one HCV protein has been replaced by a
fragment of the genome of another hepatitis C virus strain
5 which encodes the corresponding protein.

10. The nucleic acid molecule of claim 9,
wherein the protein is selected from the group consisting
of E1, E2 or NS4 proteins.

11. The nucleic acid molecule of claim 1,
wherein a fragment of the molecule encoding all or part of
an HCV protein has been deleted.

12. The nucleic acid molecule of claim 11,
wherein the HCV protein is selected from the group
15 consisting of P7, NS4B or NS5A proteins.

13. A DNA construct comprising a nucleic acid
molecule according to claims 1, 3, 5 or 8.

14. An RNA transcript of the DNA construct of
20 claim 13.

15. A cell transfected with the DNA construct
of claim 13.

16. A cell transfected with RNA transcript of
claim 14.

17. A hepatitis C virus polypeptide produced by
the cell of claim 15.

18. A hepatitis C virus polypeptide produced by
the cell of claim 16.

19. A hepatitis C virus produced by the cell of
claim 13.

20. A hepatitis C virus produced by the cell of
claim 14.

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21. A hepatitis C virus whose genome comprises a nucleic acid molecule according to claims 1, 3, 5, 6, 8, or 9.

5 22. A method for producing a hepatitis C virus comprising transfecting a host cell with the RNA transcript of claim 14.

23. A polypeptide encoded by a nucleic acid sequence according to claims 1, 2, 4 or 7 or a fragment thereof.

10 24. The polypeptide of claim 23, wherein said polypeptide is selected from the group consisting of NS3 protease, E1 protein, E2 protein or NS4 protein.

15 25. A method for assaying candidate antiviral agents for activity against HCV, comprising

a) exposing a cell containing the hepatitis C virus of claim 21 to the candidate antiviral agent; and
b) measuring the presence or absence of hepatitis C virus replication in the cell of step (a).

20 26. The method of claim 25, wherein said replication in step (b) is measured by at least one of the following: negative strand RT-PCR, quantitative RT-PCR, Western blot, immunofluorescence, or infectivity in a
25 susceptible animal.

27. A method for assaying candidate antiviral agents for activity against HCV, comprising:

a) exposing an HCV
30 protease encoded by a nucleic acid sequence according to claims 1, 2, 4, or 7, or a fragment thereof to the

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candidate antiviral agent in the presence of a protease substrate; and

b) measuring the protease activity of said protease.

5

28. The method of claim 27, wherein said HCV protease is selected from the group consisting of an NS3 domain protease, an NS3-NS4A fusion polypeptide, or an NS2-NS3 protease.

10

29. An antiviral agent identified as having antiviral activity for HCV by the method of claim 25.

30. An antiviral agent identified as having antiviral activity for HCV by the method of claim 27.

31. Antibody to the polypeptide of claim 23.

15

32. Antibody to the hepatitis C virus of claim

21.

33. A method for determining the susceptibility of cells *in vitro* to support HCV infection, comprising the steps of:

20

a. growing animal cells in vitro;

b. transfecting into said cells the nucleic acid of claim 1; and

25

c. determining if said cells show indicia of HCV replication.

34. The method according to claim 33, wherein said cells are human cells.

30

35. A cassette vector for cloning viral genomes, comprising, inserted therein, the nucleic acid sequence according to claim 2, said vector reading in the

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correct phase for the expression of said inserted sequence and having an active promoter sequence upstream thereof.

36. The cassette vector of claim 35, wherein the cassette vector is produced from plasmid pCV.

5

37. The cassette vector of claim 35, wherein the vector also contains one or more expressible marker genes.

10

38. The cassette vector of claim 35, wherein the inserted DNA sequence contains at least one ORF of the HCV genome from any strain.

39. The cassette vector of claim 35, wherein the promoter is a bacterial promoter.

15

40. A composition comprising a polypeptide of claim 23 suspended in a suitable amount of a pharmaceutically acceptable diluent or excipient.

20

41. A method for treating hepatitis C viral infection comprising the administration to a animal in need thereof of a clinically effective amount of the composition of claim 40.

25

42. A composition comprising a nucleic acid molecule of claim 1 suspended in a suitable amount of a pharmaceutically acceptable diluent or excipient.

30

43. A method for treating hepatitis C viral infection comprising the administration to an animal in need thereof of a clinically effective amount of the composition of claim 42.

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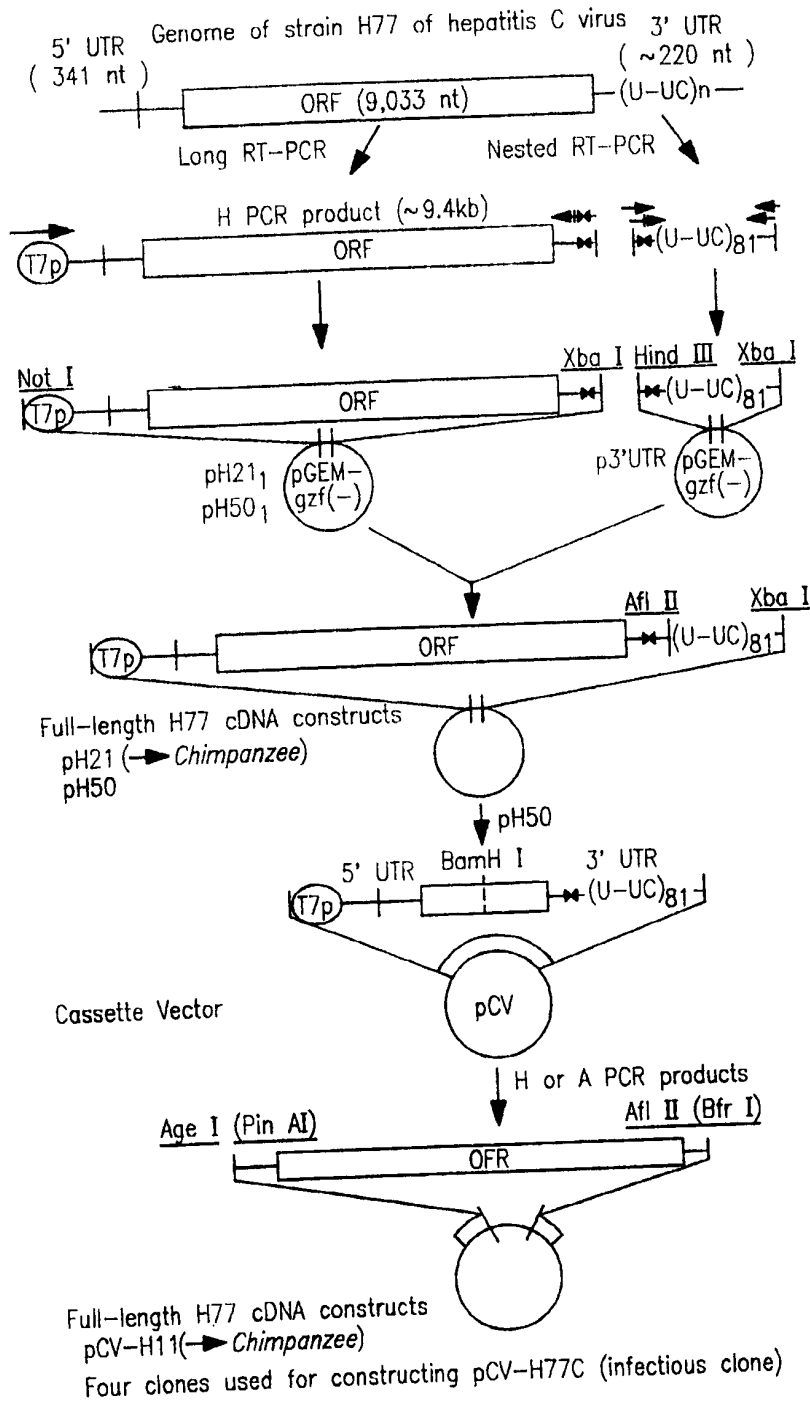


FIG. 1

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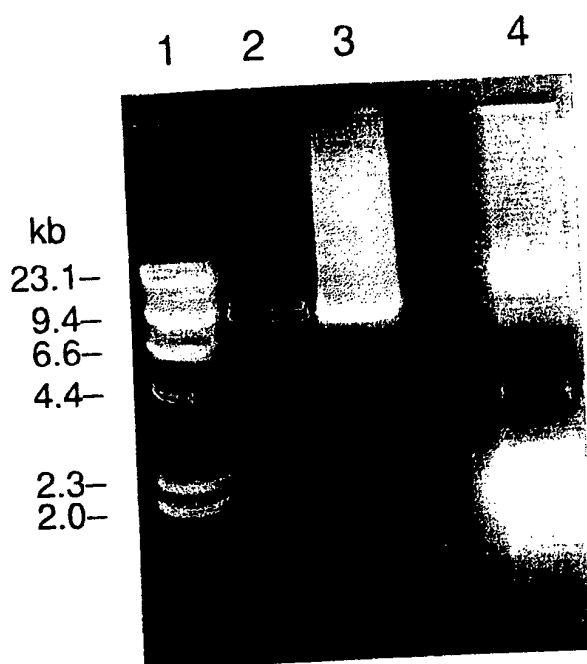


FIG. 2

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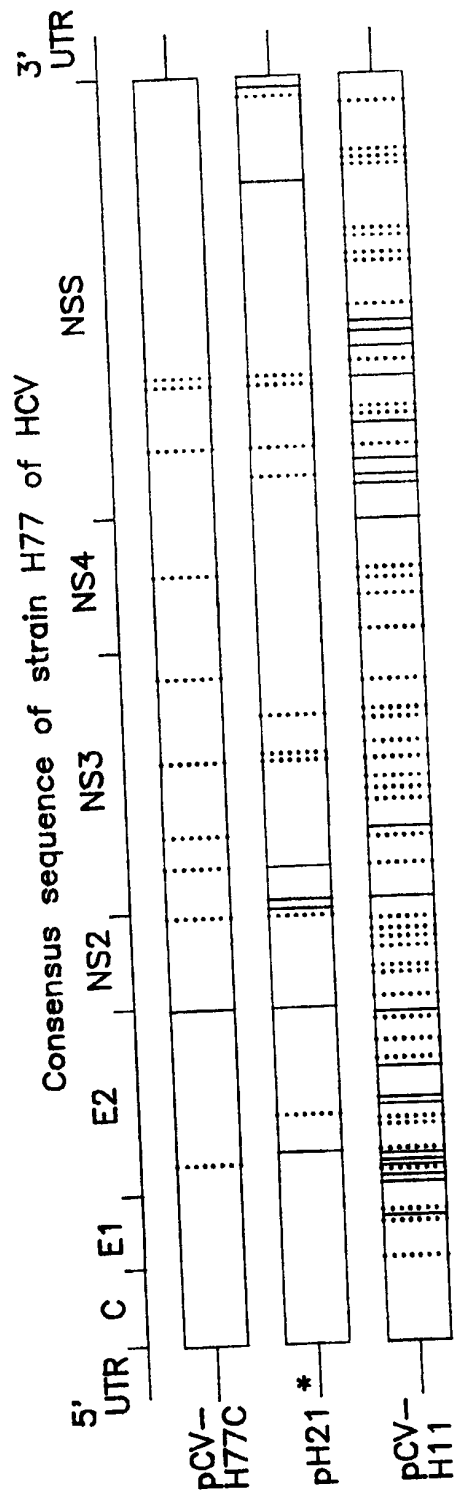


FIG. 3

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H77C

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
GCCAGCCCCC	TGATGGGGGC	GACACTCCAC	CATGAATCAC	TCCCCTGTGA	50
GGAACACTAG	TCTTCAAGCA	GAAAGCGTCT	AGCCATGGCG	TTAGTATGAG	100
TGTGCTGCAG	CCTCCAGGAC	CCCCCTCC	GGGAGAGCCA	TAGTGGTCTG	150
CGGAACCGGT	GAGTACACCG	GAATTGCCAG	GACGACCGGG	TCCTTTCTTG	200
GATAAACCCG	CTCAATGCCT	GGAGATTITG	GCGTGGCCCC	GCAAGACTGC	250
TAGCCGAGTA	GTGTGGGGTC	GCGAAAGGCC	TTGTGGTACT	GCCTGATAGG	300
GTGCTTGGGA	GTGCCCCGGG	AGGTCTCGTA	GACCGTGCAC	CATGAGCAAG	350
AATCCATAAC	CTCAAAGAAA	AACCAAACGT	AACACCAACC	GTGCCCCACA	400
GGACGTCAAG	TTCGCGGGTG	GCGGTTCAGT	CGTTGGTGGG	GTATTCTTGT	450
TGCGCGCGAG	GGGCCCCAGA	TTGGGTGTGC	GCGGACGAG	GAAGACTTCC	500
GAGCGGTGCG	AACCTCGAGG	TAGACGTACG	CCTATCCCCA	AGGCAAGTGG	550
GCCCGAGGGC	AGGAOCTGGG	CTCAGCCCCG	GTACCCCTTG	CCCCCTCTAT	600
GCAATGAGGG	TTGCGGGTGG	GCGGGATGGC	TCCTGTCTCC	CCGTGGCTCT	650
CGGCCCTAGCT	GGGGCCCCAC	AGACCCCCCG	CGTAGGTGCG	GCAATTITGGG	700
TAAGGTCAATC	GATACCCCTTA	CGTGCGGCTT	CGCCGACCTC	ATGGGGTACA	750
TACCGCTCGT	CGGCGCCCCT	CTTGAGGGCG	CTGCCAGGGC	CCTGGCGCAT	800
GGCGTCCGGG	TTCTGGAAGA	CGGCGTGAAC	TATGCAACAG	GGAACCTTCC	850
TGGTGTGCTCT	TTCTCTATCT	TCCTTCTGGC	CCTGCTCTCT	TGCTGACTTG	900
TGCCCCGCTTC	AGCCTACCAA	GTGCGCAATT	CCTCGGGGCT	TTACCATGTC	950
ACCAATGATT	GCCCTAACTC	GAGTATTGTG	TACGAGGGGG	CCGATGCCAT	1000
CCTGCACACT	CCGGGGTGTG	TCCCTTGCGT	TGCGAGGGGT	AACGCTCGA	1050
GGTGTGTGGT	GGCGGTGACC	CCACCGGTGG	CCACCAGGGA	CGGCAAACTC	1100
CCCAACAACG	AGCTTCGACG	TCATATCGAT	CTGCTTGTGG	GGAGCGGCAC	1150
CCTCTGCTGG	GCCCTCTACG	TGGGGGACCT	GTGCGGGTCT	GTCTTTCTTG	1200
TTGGTCAACT	GTTTACCTTC	TCTCCCAGGC	GCCACTGGAC	GACGCAAGAC	1250
TGCAATTGTT	CTATCTATCC	CGGCCATATA	AAGGGTCATC	GCATGGCATG	1300
GGATATGATG	ATGAACCTGG	CCCCTAAGGC	AGCGTTGGTG	GTAGCTCAGC	1350
TGCTCCGGAT	CCCAACAAGC	ATCATGGACA	TGATCGCTGG	TGCTCACTGG	1400
GGAGTCCCTGG	CGGGCATAGC	GTATTTCTCC	ATGGTGGGGA	ACTGGGCGAA	1450
GGTCCCTGGTA	GIGCTGTCTG	TATTTTGCCG	CGTCGACGGG	GAAACCCACG	1500
TCACCGGGGG	AAATGCGGGC	CGCACCAACG	CTGGGCTTGT	TGGTCTCCTT	1550
ACACCAAGCG	CCAAGCAGAA	CATCCAACCT	ATCAACACCA	ACGGCAGTTG	1600
GCACATCAAT	AGCACGGCCT	TGAATTGCAA	TGAAAGCCTT	AACACCGGCT	1650
GGTTAGCAGG	GCTCTTCTAT	CAACACAAAT	TCAACTCTTC	AGGCTGTCTT	1700
GAGAGGTTGG	CCAGCTGCCG	ACGCCCTTAC	GATTTTGGCC	AGGGCTGGGG	1750
TCCTATCAGT	TATGCCAACG	GAAGCGGCCT	CGACGAACGC	CCCTACTGCT	1800
GGCACTACCC	TCCAAGACCT	TGTGGCAATT	TGCCCCGAAA	GAGCGTGTGT	1850
GGCCCGGTAT	ATTGCTTCAC	TCCAGCCCC	GTGGTGGTGG	GAACGACCGA	1900

FIG. 4A

H77C

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
CAGGTCGGGC	GCGCTACCT	ACAGCTGGGG	TGCAAATGAT	ACGGATGICT	1950
TGTCCTTAA	CAACACCAGG	CCACCGCTGG	GCAATIGGTT	CGGTIGTACC	2000
TGGATGAAT	CAACTGGATT	CACCAAAGTG	TGCGGAGCGC	CCCCGTGIGT	2050
CATCGGAGGG	GIGGGCAACA	ACACCTTGCT	CTGCCCCACT	GATTGCTTCC	2100
GCAAACATCC	GGAAGCCACA	TACTCTCGGT	GCGGCTCCGG	TCCCIGGATT	2150
ACACCCAGGT	GCATGGTCCA	CTACCCGAT	AGGCTTTGGC	ACTATCCTTG	2200
TACCATCAAT	TACACCATAT	TCAAAGTCAG	GATGTACGTG	GGAGGGGTGG	2250
AGCACAGGCT	GGAAGCGGCC	TGCAACTGGA	CGCGGGGCGA	AOGCTGTGAT	2300
CTGGAAGACA	GGGACAGGTC	CGAGCTCAGC	CGGTIGCTGC	TGTCCACCAC	2350
ACAGTGGCAG	GTCCTTCGGT	GTTCCTTCAC	GACCCTGCCA	GCCTTGTCCA	2400
CCGGCCTCAT	CCACCTCCAC	CAGAACATTG	TGGACGTCCA	GTACTTGTAC	2450
GGGGTAGGGT	CAAGCATGGC	GTCCTGGGCC	ATTAAAGTGG	AGTACGTGGT	2500
TCTCCTGTTC	CTTCGTCTTG	CAGACGCGG	CGTCTGCTCC	TGCTTGTGGA	2550
TGATGTACT	CATATCCCAA	GCGGAGGCGG	CTTTGGAGAA	CCTCGTAATA	2600
CTCAATGCAG	CATCCCTGGC	CGGACGCGAC	GGTCTTGTGT	CCTTCCTCGT	2650
GTTCCTCTGC	TTTGGGTGGT	ATCTGAAGGG	TAGGTGGGTG	CCCGGAGCGG	2700
TCTACGCCCT	CTACGGGATG	TGGCCTCTCC	TCCGTCTCCT	GCTGGCGTTG	2750
CCTCAGCGGG	CATACGCACT	GGACACGGAG	GIGGCCGCGT	CGTGTGGCGG	2800
CGTTGTCTTT	GTCGGGTAA	TGGCGCTGAC	TCTGTGCGCA	TATTACAAGC	2850
GCTATATCAG	CTGGTGCATG	TGGTGGCTTC	AGTATTTTCT	GACCAGAGTA	2900
GAAGCGCAAC	TGCACGTGTG	GGTCCCCCCC	CTCAACGTCC	GGGGGGGGCG	2950
CGATGCGGTC	ATCTTACTCA	TGTGTGTAGT	ACACCCGACC	CTGGTATTTG	3000
ACATCACCAA	ACTACTCCTG	GCCATCTTGG	GACCCCTTTG	GATTCTTCAA	3050
GCCAGTTTGC	TAAAGTCCC	CTACTTGGTG	CGGTTTCAAG	GCCTTCTCCG	3100
GATCTGGCGG	CTAGCGCGGA	AGATAGCGGG	AGGTCAATTAC	GTGCAAATGG	3150
CCATCATCAA	GTTAGGGGGG	CTTACTGGCA	CCTATGTGTA	TAAACATCTC	3200
ACCCCTCTTC	GAGACTGGGC	GCACAACGGC	CTGCGAGATC	TGGCCGTGGC	3250
TGTGGAACCA	GTCGTCTTCT	CCCGAATGGA	GACCAAGCTC	ATCAAGTGGG	3300
GGGCAGATAC	CGCCGCGTGC	GGTGACATCA	TCAAOGGCTT	GCCCGTCTCT	3350
GCCCGTAGGG	GCCAGGAGAT	ACTGCTTGGG	CCAGCCGACG	GAATGGTCTC	3400
CAAGGGGTGG	AGGTGTCTGG	CGCCCATCAC	GGCGTACGCC	CAGCAGACGA	3450
GAGGCCTCCT	AGGGTGTATA	ATCACCAGCC	TGACTGGCCG	GGACAAAAC	3500
CAAGTGGAGG	GTGAGGTCCA	GATCGTGTCA	ACTGCTTACC	AAACCTTCTT	3550
GGCAACGTGC	ATCAATGGGG	TATGCTGGAC	TGTCTACCAC	GGGGCCGGAA	3600
CGAGGACCAT	CGCATCACCC	AAGGGTCTCT	TCATCCAGAT	GTATACCAAT	3650
GTGGACCAAG	ACCTTGTGGG	CTGGCCCGCT	CCTCAAGGTT	CCCGCTCATT	3700
GACACCTGT	ACCTGCGGCT	CCTGGACCT	TACCTGGTC	ACGAGGCACG	3750
CCGATGTAT	TCCCGTGGC	CGGCGAGGTG	ATAGCAGGGG	TAGCCTGCTT	3800

FIG. 4B

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H77C

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
TCGCCCCGGC	CCATTTCCTA	CTTGAAAGGC	TCCTCGGGGG	GTCCGCTGTT	3850
GTGCCCCGGC	GGACACGGCG	TGGGCTTATT	CAGGGCCGGG	GTGTGCACCC	3900
GTGGAGTGGC	TAAAGCGGTG	GACTTTATCC	CTGTGGAGAA	CCTAGGGACA	3950
ACCATGAGAT	CCCCGGTGTT	CACGGACAAC	TCCTCTCCAC	CAGCAGTGCC	4000
CCAGAGCTTC	CAGGTGGGCC	ACCTGCATGC	TCCCACGGGC	AGCGGTAGA	4050
GCACCAAGGT	CCCGGCTGGG	TACGCAGGCC	AGGGCTACAA	GGTGTGGTG	4100
CTCAACCCCT	CTGTGTCTGC	AACGCTGGGC	TTTGGTGCTT	ACATGTCCAA	4150
GGCCCATGGG	GTGTATCCTA	ATATCAGGAC	CGGGGTGAGA	ACAATTACCA	4200
CTGGCAGCCC	CATCAGGTAC	TCCACCTACG	GCAAGTTCC	TGCGAAGGC	4250
GGGTGCTCAG	GAGGTGCTTA	TGACATAATA	ATTTGTGACG	AGTGCCACTC	4300
CACGGATGCC	ACATCCATCT	TGGGCATCGG	CACGTGCTTT	GACCAAGCAG	4350
AGACTGCGGG	GGCGAGACTG	GTGTGTCTCG	CCACTGCTAC	CCCTCCGGGC	4400
TCCGTCACTG	TGTCCCATCC	TAACATOGAG	GAGGTGTCTC	TGTCCACCAC	4450
CGGAGAGATC	CCCTTTTACG	GCAAGGCTAT	CCCCCTCGAG	GTGATCAAGG	4500
GGGGAAGACA	TCTCATCTTC	TGCCACTCAA	AGAAGAAGTG	CGACGAGCTC	4550
GCCGCGAAGC	TGGTCCGATT	GGGCATCAAT	GCCGTGGCCT	ACTACCGGGG	4600
TCTTGACGTG	TCTGTCTATC	CGACCAGCGG	CGATGTGTGC	GTGTGTCTGA	4650
CCGATGCTCT	CATGACTGGC	TTTACCGGGG	ACTTCGACTC	TGTGTATAGAC	4700
TGCAACACGT	GTGTCACTCA	GACAGTCGAT	TTTACGCTTG	ACCTTACCTT	4750
TACCAATTGAG	ACAACCACGC	TCCCCCAGGA	TGCTGTCTCC	AGGACTCAAC	4800
GCCGGGGCAG	GACTGGCAGG	GGGAAGCCAG	GCATCTATAG	ATTTGTGGCA	4850
CCGGGGGAGC	GCCCCCTCGG	CATGTTTCGAC	TGTTCCGTCC	TCTGTGAGTG	4900
CTATGACCGG	GGCTGTGCTT	GGTATGAGCT	CACGCCCGCC	GAGACTACAG	4950
TTAGGCTACG	AGCGTACATG	AACACCCCGG	GGCTTCCCGT	GTGCCAGGAC	5000
CATCTTGAAT	TTTGGGAGGG	CGTCTTTACG	GGCTCACTC	ATATAGATGC	5050
CCACTTTTTTA	TCCCAGACAA	AGCAGAGTGG	GGAGAACCTT	CCTTACCTGG	5100
TAGCGTACCA	AGCCACCGTG	TGGGCTAGGG	CTCAAGGCCC	TCCCCCATCG	5150
TGGGACCAGA	TGTGGAAGTG	TTTGATCCGC	CTTAAACCCA	CCCTCATGGG	5200
GCCAACACCC	CTGCTATACA	GACTGGGGGC	TGTTTCAGAA	GAAGTCACCC	5250
TGACGCACCC	AATCACCAAA	TACATCATGA	CATGCATGTC	GGCCGACCTG	5300
GAGGTGCTCA	CGAGCACCTG	GGTGCTCGTT	GGGGGGGTCC	TGGCTGCTCT	5350
GGCCCGGTAT	TGCTGTGCAA	CAGGCTGGGT	GGTCATAGTG	GGCAGGATCG	5400
TCTTGTCCGG	GAAGCCGGCA	ATTATACCTG	ACAGGGAGGT	TCTCTACCAG	5450
GAGTTCGATG	AGATGGAAGA	GTGCTCTCAG	CACCTACCGT	ACATCGAGCA	5500
AGGGATGATG	CTGGCTGAGC	AGTTCAGCA	GAAGGCCCTC	GGCTCCTGTC	5550
AGACCGCGTC	CCGCCATGCA	GAGGTATATCA	CCCCGTCTGT	CCAGACCAAC	5600
TGGCAGAAAC	TGGAGGTCTT	TGGGGCGAAG	CACATGTGGA	ATTTCATCAG	5650
TGGGATACAA	TACTTGGCGG	GCCTGTCAAC	GCTGCTGGT	AACCCCGCCA	5700

FIG. 4C

SUBSTITUTE SHEET (RULE 26)

H77C

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
TTGCTTCATT	GATGGCTTTT	ACAGCTGCCG	TCACCAGCCC	ACTAACCACT	5750
GGCCAAACCC	TCTCTTCAA	CATATIGGGG	GGGTGGGIGG	CTGCCCAGCT	5800
CGCCGCCCCC	GGTGCCGCTA	CTGCCCTTGT	GGGTGCTGGC	CTAGCTGGCG	5850
CCGCCATCGG	CAGCGTIGGA	CTGGGGAAGG	TCTCTGTGGA	CATTCTTGCA	5900
GGGTATGGCG	CGGGCGTGGC	GGGAGCTCTT	GTAGCATTCA	AGATCATGAG	5950
CGGTGAGGTC	CCCTCCACGG	AGGACCTGGT	CAATCTGCTG	CCCGCCATCC	6000
TCTCGCCCTGG	AGCCCTGTGA	GTGGGTGTGG	TCTGGGCAGC	AATACTGGCG	6050
CGGCAAGTTG	GCCCCGGGGA	GGGGGCAGTG	CAATGGATGA	ACCGGCTAAT	6100
AGCCCTGCGC	TCCCCGGGGG	ACCATGTTTC	CCCCACGCAC	TACGTGCGCG	6150
AGAGCGATGC	AGCCGCCCCG	GTCACTGCCA	TACTCAGCAG	CCTCACTGTA	6200
AACCAGCTCC	TGAGGCGACT	GCATCAGTGG	ATAAGCTCGG	AGTGTACCCAC	6250
TCCATGCTCC	GGTTCCTGGC	TAAGGGACAT	CTGGGACTGG	ATATGCGAGG	6300
TGCTGAGCGA	CTTTAAGACC	TGGCTGAAAG	CCAAGCTCAT	GCCACAACCTG	6350
CCTGGGATTTC	CCTTTGTGTG	CTGCCAGCGC	GGGTATAGGG	GGGTCTGGCG	6400
AGGAGACGGC	ATTATGCACA	CTCGCTGCCA	CTGTGGAGCT	GAGATCACTG	6900
GACATGTCAA	AAACGGGACG	ATGAGGATCG	TGGTTCCTAG	GACCTGCAGG	6950
AACATGTGGA	GTGGGACGTT	CCCCATTAAC	GCCTACACCA	CGGGCCCCCTG	6550
TACTCCCCCTT	CCTGCCGCCG	ACTATAAGTT	CGCGCTGTGG	AGGGTGTCTG	6600
CAGAGGAATA	CGTGGAGATA	AGGCGGGTGG	GGGACTTCCA	CTACGTATCG	6650
GGTATGACTA	CTGACAATCT	TAAATGCCCG	TGCCAGATCC	CATCGCCCCGA	6700
ATTTTTCACA	GAATTGGACG	GGGTGCGGCT	ACACAGGTTT	GCGCCCCCTT	6750
GCAAGCCCTT	GCTGCGGGAG	GAGGTATCAT	TCAGAGTAGG	ACTCCACGAG	6800
TACCGGGTGG	GGTCCCAATT	ACCTTGCGAG	CCCGAACCGG	ACGTAGCCGT	6850
GTGTACGTCC	ATGCTCACTG	ATCCCTCCCA	TATAACAGCA	GAGGCGGCGG	6900
GGAGAAGGTT	GGGAGAGGGG	TCACCCCTTT	CTATGGCCAG	CTCCTGGGCT	6950
AGCCAGCTGT	CCGCTCCATC	TCTCAAGCCA	ACTTGCACCG	CCAACCATGA	7000
CTCCCTTGAC	GCCGAGCTCA	TAGAGGCTAA	CCTCCTGTGG	AGGCAGGAGA	7050
TGGGCGGCAA	CATCACCAGG	GTTGAGTCAG	AGAACAAAGT	GGTGATTCTG	7100
GACTCCTTCG	ATCCGCTTGT	GGCAGAGGAG	GATGAGCGGG	AGGTCTCCGT	7150
ACCTGCAGAA	ATTCTGCGGA	AGTCTCGGAG	ATTGCGCCGG	GCCCTGCCCG	7200
TCTGGGCGCG	GCCGACTTAC	AACCCCCCGC	TAGTAGAGAC	GTGGAAAAAG	7250
CCTGACTACG	AACCACCTGT	GGTCCATGGC	TGCCCCGTAC	CACCTCCACG	7300
GTCCCCCTCCT	GTCCTCCGCG	CTCGGAAAAA	GCGTACGGTG	GTCTTCAACG	7350
AATCAACCCCT	ATCTACTGCC	TTGGCCGAGC	TTGCCACCAA	AAGTTTIGGC	7400
AGCTCCTCAA	CTTCCGGCAT	TACGGGCGAC	AATACGACAA	CATCCTCTGA	7450
GCCCCCCCCCT	TCTGGCTGCC	CCCCCGACTC	CGAAGTTGAG	TCCATATCTT	7500
CCATGCCCCCC	CCTGGAGGGG	GAGCCTGGGG	ATCCGGATCT	CAGCGACGGG	7550
TCATGGTCCA	CGGTCACTAG	TGGGGCCGAC	ACGGAAGATG	TGCTGTGCTG	7600

FIG. 4D

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H77C

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
CTCAATGTCT	TATTCCTGGA	CAGGCGCACT	CGTCACCCCG	TGCGCTGCGG	7650
AAGAACAAAA	ACTGCCCCATC	AACGCACTGA	GCAACTCGTT	GCTACGCCAT	7700
CACAATCTGG	TGTATTCCAC	CACCTTCACG	AGTGCTTGCC	AAAGGCAGAA	7750
GAAAGTCACA	TTTGACAGAC	TGCAAGTTCT	GGACAGCCAT	TACCAGGACG	7800
TGCTCAAGGA	GGTCAAAGCA	GCGGOGTCAA	AAGTGAAGGC	TAACTTGCTA	7850
TCGGTAGAGG	AAGCTTGCGAG	CCTGAGGCCC	CCACATTGAG	CCAAATOCAG	7900
GTTTGGCTAT	GGGGCAAAAG	ACGTCCGTTG	CCATCCGAGA	AAGGCCGTTAG	7950
CCCACATCAA	CTCCGTTGTTG	AAAGACCTTC	TGGAAGACAG	TGTAACACCA	8000
ATAGACACTA	CCATCATGGC	CAAGAAGCAG	GTTTTCTGGG	TTCAGCCTGA	8050
GAAGGGGGGT	CGTAAGCCAG	CTCGTCTCAT	CGTGTTCCCC	GACCTGGGCG	8100
TGCGCGTGTG	CGAGAAGATG	GCCCTGTACG	ACGTGGTTAG	CAAGCTCCCC	8150
CTGGCCGTGA	TGGGAAGCTC	CTACGGATTG	CAATACTCAC	CAGGACAGCG	8200
GGTTGAATTG	CTCGTGCAAG	CGTGGAAGTC	CAAGAAGACC	CCGATGGGGT	8250
TCTCGTATGA	TACCCGCTGT	TTTGACTCCA	CAGTCACTGA	GAGCGACATC	8300
CGTACCGAGG	AGGCAATTTA	CCAATGTTGT	GACCTGGACC	CCCAAGCCCC	8350
CGTGGCCATC	AAGTCCCTCA	CTGAGAGGCT	TTATGTTGGG	GGCCCTCTTA	8400
CCAATTCAAG	GGGGGAAAC	TGCGGCTACC	GCAGGTGCCC	CGCGAGCGGC	8450
GTACTGACAA	CTAGCTGTGG	TAACACCTTC	ACTTGCTTAC	TCAAGGCCCG	8500
GGCAGCCTGT	CGAGCCGCGAG	GGCTCCAGGA	CTGCACCATG	CTCGTGTGTG	8550
GCGACGACTT	AGTCGTTATC	TGTGAAAGTG	CGGGGGTCCA	GGAGGACGGG	8600
GCGAGCCTGA	GAGCCTTCAC	GGAGGCTATG	ACCAGGTACT	CCGCCCCCCC	8650
CGGGGACCCC	CCACAACCAG	AATACGACTT	GGAGCTTATA	ACATCATGCT	8700
CCTCCAACGT	GTCAGTGGCC	CACGACGGCG	CTGGAAAGAG	GGTCTACTAC	8750
CTTACCCGTG	ACCCCTACAAC	CCCCCTGGCG	AGAGCCGGGT	GGGAGACAGC	8800
AAGACACACT	CCAGTCAATT	CCTGGCTAGG	CAACATAATC	ATGTTTGGCC	8850
CCACACTGTG	GGCGAGGATG	ATACTGATGA	CCCATTTCTT	TAGCGTCTTC	8900
ATAGCCAGGG	ATCAGCTTGA	ACAGGCTCTT	AACGTGTGAG	TCTACGGAGC	8950
CTGCTACTCC	ATAGAACCAC	TGGATCTACC	TCCAATCATT	CAAAGACTCC	9000
ATGGCCCTCAG	CGCATTTTCA	CTCCACAGTT	ACTCTCCAGG	TGAAATCAAT	9050
AGGGTGGCCG	CATGCCCTCAG	AAACTTTGGG	GTCCCGCCCT	TGCGAGCTTG	9100
GAGACACCGG	GCCCCGAGCG	TCCGCGCTAG	GCTTCTGTCC	AGAGGAGGCA	9150
GGGCTGCCAT	ATGTGGCAAG	TACCTCTTCA	ACTGGGCAGT	AAGAACAAGG	9200
CTCAAACTCA	CTCCAATAGC	GGCCGCTGGC	CGGCTGGACT	TGTCCGGTTG	9250
GTTACAGGCT	GGCTACAGCG	GGGAGACAT	TTATCACAGC	GIGTCTCATG	9300
CCCGGCCCCG	CTGGTTCTGG	TTTTCGCTAC	TCTTGCTGGC	TGCAGGGGTA	9350
GGCATCTACC	TCTTCCCCAA	CCGATGAAGG	TTGGGGTAAA	CACCTCCGGC	9400
TCTTAAGCCA	TTTCCGTGTT	TTTTTTTTTT	TTTTTTTTTT	TTTTTCTTTT	9450
TTTTTTTCTT	TCTTTCCCTT	CTTTTTTTCC	TTCTTTTTTC	CCTTCTTTAA	9500

FIG. 4E

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H77C

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
TGGTGGCTCC	ATCTTAGCCC	TAGTCACGGC	TAGCTGTGAA	AGGTCCGTGA	9550
GGCGCATGAC	TGCAGAGAGT	GCTGATACTG	GOCTCTCTGC	AGATCATGT	9599

FIG. 4F

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H77C

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
MSINPKPQK	TKRNINRRPQ	DVKFPGGGQI	VGGVYLLPRR	GPRLGVRATR	50
KTSESRQPRG	RRQPIPKARR	PEGRIWAQPG	YFWPLYGNEG	CGWAGALLSP	100
RGSRPSWGPT	DPRRRSRNLG	KVIDILTQGF	ADLMGYIPLV	GAPLGGAARA	150
LAHGVRVLED	GVNYATGNLP	GCSFSIFLLA	LLSCLTVPAS	AYQVRNSSGL	200
YHVINDCENS	SIVYEADAIA	LHTPGCVPCV	REGNASRCWV	AVTPTIVATRD	250
GKLEPTTQLRR	HIDLLVGSAT	LCSALYVGDL	CGSVFLVGQL	FTFSPPRRHWT	300
TQDCNCSTYP	GHTTGHMAW	DMMNWSPTA	ALVVAQLLRI	PQAIMDMIAG	350
AHWGVLGATA	YFSMVGWAK	VLVLLLLFAG	VDAETHVTGG	NAGRTTAGLV	400
GLLTGAKQN	IQLININGSW	HINSTALNCH	ESLNTGWLAG	LFYQHKFNSS	450
GCPERLASCR	RLIDEAQGWG	PLSYANGSGL	DERPYCWHYP	PRPGIVPAK	500
SVOGPVYCFE	PSPVVVGTTD	RSGAPTYSWG	ANDIDVFEVN	NTRPPLGNWF	550
GCTWMNSTGF	TKVCGAPPCV	IGGVGNITLL	CPTDCFRKHP	EATYSRCGSG	600
PWITPRQMD	YPYRLWHYPC	TINYTIFKVR	MYVGGVEHRL	EACNWIWGE	650
RCDLEDNRDS	ELSPLLLSTT	QWQVLPCSFT	TLPALSTGLI	HLHQNIQVQ	700
YLYGVGSSIA	SWAIKWEYVW	LLFLLLADAR	VCSCILWMLL	ISQAEAALEN	750
LVLINAASLA	GTHGLVSFLV	FFCFAWYILKG	RWVPGAVYAL	YGMWPLLLLL	800
LALPQRAYAL	DTEVAASCGG	VVLVGLMALT	LSPYYKRYIS	WCMWWLQYFL	850
TRVEAQLHWV	VPPLNVRGGR	DAVILLMCW	HPTLVFDITK	LLLAIFGPLW	900
ILQASLLKVP	YFVRVQGLLR	ICALARKIAG	GHYVQMAIHK	LGALITGYVY	950
NHLTFLRDWA	HNGLRDLAVA	VEPVVFSRME	TKLITWGADT	AACGDIINGL	1000
PVSARRQOEI	LLGPADGMVS	KGWRLLAPIT	AYAQQTRGLL	GCIITSLTGR	1050
DKNQVEGEVQ	IVSTATQTFL	ATCINGVCWT	VYHGAGTRTI	ASPKGFVIQM	1100
YTNVDQDLVG	WPAPQGSRL	TPCTCGSSDL	YLVTRHADVI	FVRRRGDSRG	1150
SLLSPRPISY	LKGSSGGPLL	CPAGHAVGLF	RAAVCTRGVA	KAVDFIPVEN	1200
LGTIMRSPVF	TDNSSPPAVP	QSFOVAHLHA	PTGSGKSTKV	PAAYAAQGYK	1250
VLVLNPSVAA	TLGFGAYMSK	AHGVDENIPT	GVRTITITGSP	ITYSTYGFKL	1300
ADGGCSGGAY	DIICDECHS	TDATSIIGIG	TVLDQAETAG	ARLVLATAT	1350
PPGSVIVSHP	NIEEVALSTT	GEIPFYGKAI	PLEVIKGRH	LIFCHSKKKC	1400
DELAACKLVAL	GINAVAYYRG	LDVSVIPTSG	DVVVSTDAL	MITFTGDFDS	1450
VIDCNTCVTQ	TVDFSLDPTF	TIETTTILPOD	AVSRITQRRGR	TGRGKPGIYR	1500
FVAPGERPSG	MFDSSVLCEC	YDAGCAWYEL	TPAETTVRLR	AYMNTPGLEV	1550
QQDHLEFWEG	VFTGLTHIDA	HFLSQTKQSG	ENFPYLVAYQ	ATVCARAQAP	1600
PPSWDQMAKC	LIRLKPILHG	PTPLLYRLGA	VQNEVTILTHP	ITKYIMTQMS	1650
ADLEVVISIW	VLVGGVLAAL	AAYCLSTGCV	VIVGRIVLSG	KPAIIPDREV	1700
LYQEFDEMEE	CSQHLPTYEQ	GMLAEQFKQ	KALGLLQIAS	RHAEVITPAV	1750
QTNWQKLEVF	WAKHMANFTS	GIQYLAGLST	LPGNPATIASL	MAFTAAVTSP	1800
LITIGQTLLEN	ILGGWAAQL	AAPGAATAFV	GAGLAGAAIG	SVGLGKVLVD	1850
ILAGYGAGVA	GALVAFKIMS	GEVPSTEDLV	NLLPAILSPG	ALVVGWVCAA	1900

FIG. 4G

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H77C

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
ILRRHVGPGE	GAVQWMNRLI	AFASRGNHVS	PIHYVPESDA	AARVTAILSS	1950
LIVTQLLRRL	HQWISSECTT	PCSGSWLRDI	WDWICEVLSD	FKIWLKAKLM	2000
PQLPGIPFVS	CQRGYRGVWR	GDGIMHIRCH	CGAEITGHVK	NGIMRIVGPR	2050
TCRNMMMSGTF	PINAYTTGPC	TPLPAPNYKF	ALWRVSAEEY	VEIRRVGDFH	2100
YVSGMITDNL	KCPQIQPSPE	FFTELDGVRL	HRFAPPCKPL	LREEVSFRVG	2150
LHEYPVGSQI	PCEPEPDVAV	LITSMITDPSH	ITAEFAAGRRL	ARGSPPSMAS	2200
SSASQISAPS	LKATCTANHD	SPDAELIEAN	LLWRQEMGGN	ITRVESENKV	2250
VILDSFDPLV	AEEDEREVS	PAETLRKSRR	FARALEVWAR	PDYNPFIVET	2300
WKKPDYEPFV	VHGCPPLPPR	SPFVPPPRKK	RTVVLTESTL	STALAEIATK	2350
SFGSSSTSGI	TGDNITTSSE	PAPSGCPPDS	DVESYSSMPP	LEGEFGDPDL	2400
SDGSWSTVSS	GADTEDVCC	SMSYSWIGAL	VTFCAEEQK	LPINALSNSL	2450
LRHHNLVYST	TSRSACQOK	KVTFDRLQVL	DSHYQDLKE	VKAAASKVKA	2500
NLLSVEEACS	LTPPHSAKSK	FGYGAKDVRC	HARKAVAHIN	SVWKDILLED	2550
VTPIDITTIMA	KNEVFCVQPE	KGGRKPARLI	VFPDLGVRVC	EKMALYDVVS	2600
KLPLAVMGSS	YGFOYSPGQR	VEFLVQAWKS	KKTPMGFSYD	TRCFDSTVTE	2650
SDIRTEEATY	QCCDLDPQAR	VAIKSLITERL	YVGGPLTINSR	GENCGYRRCR	2700
ASGVLTTSCG	NILTCYIKAR	AACRAAGLQD	CIMLVCGDDL	VVICESAGVQ	2750
EDAASLRAFT	EAMTRYSAAP	GDPPQPEYDL	ELITSCSSNV	SVAHDGAGKR	2800
VYLTDRDPTT	PLARAAMETA	RHTPVNSWL	NIIMFAPTLW	ARMILMTHFF	2850
SVLIARDQLE	QALNCEIYGA	CYSIEPLDLP	PIIQLHGLS	AFSLHSYSEF	2900
EINRVAACLR	KLGVPELRAW	RHRARSVRAR	LLSRGGRAAI	CGKYLENWAV	2950
RTKLKLTPIA	AAGRLDLGSW	FTAGYSGGDI	YHSVSHARPR	WFWFCLLLLA	3000
AGVGIIYLLN	R				3011

FIG. 4H

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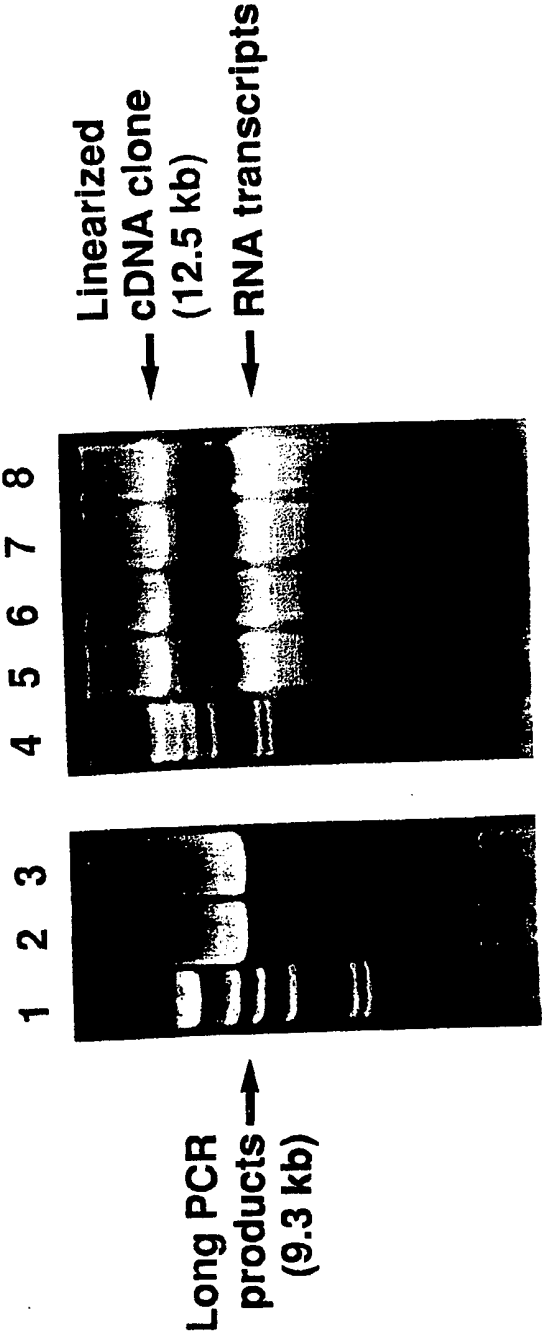


FIG. 5

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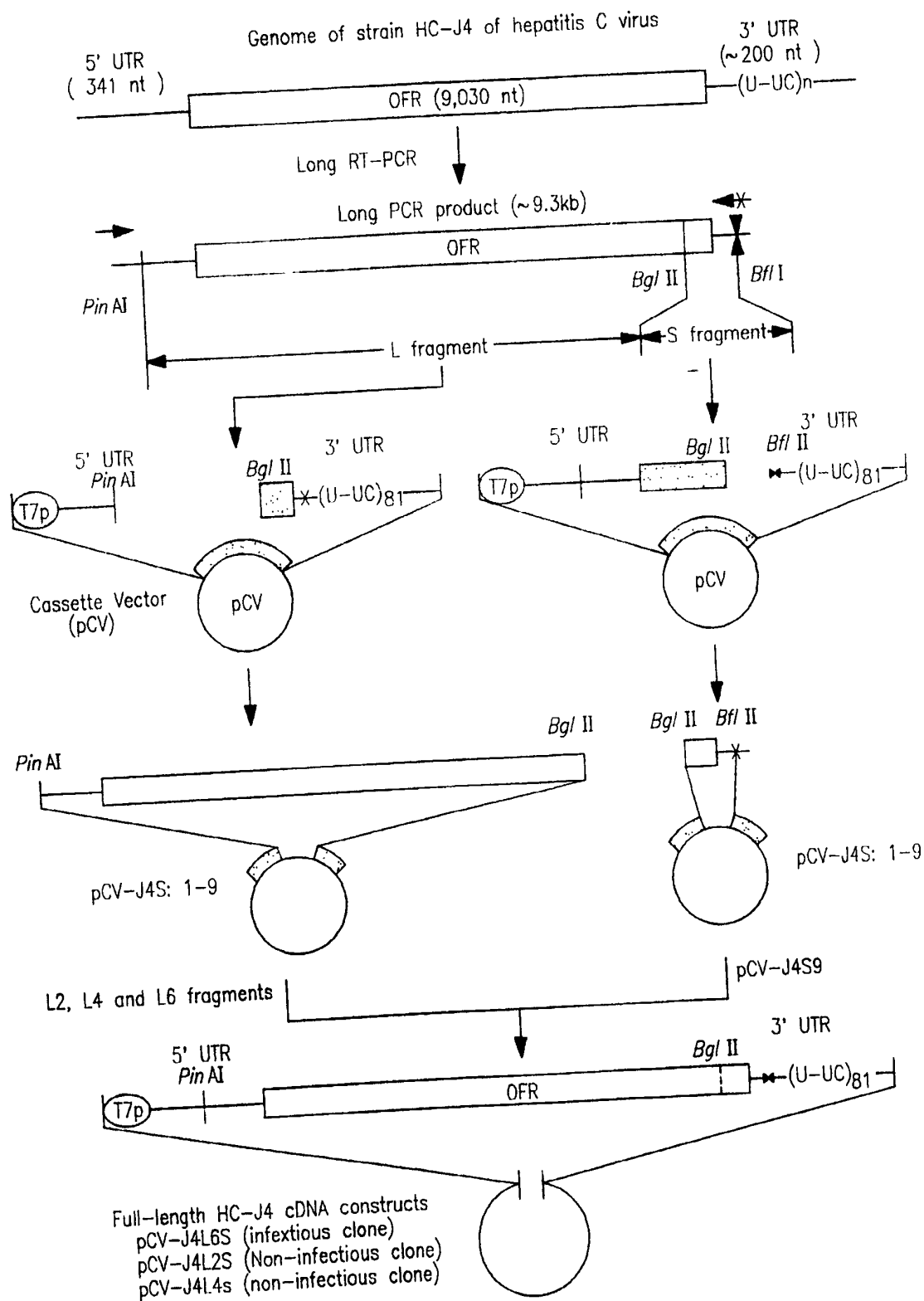


FIG. 6
SUBSTITUTE SHEET (RULE 26)

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	L fragment	Cons-p9	L1*(A)	L2(A)	L6(A)	L8(A)	L9(A)	L3(B)	L7*(B)	L10(B)	L4(C)	Cons-D	Cons-F
Core	16	N	S	N
	36	L	.	.	.	P	L
	52	A	T	T	T	T	T	A,T
	70	R	Q	Q	Q	.	R,Q	R,Q
	189	A	T	.	.	.	A
	195	R	H	.	H	.	.	R
	231	R	.	Q	Q	Q	R
E1	233	G	A	A	A	.	.	G
	234	N	D	D	D	.	.	N
	250	N	D	.	N
	299	E	A	.	.	.	A	E
	304	C	.	V	C
	379	A	T	.	T	.	.	A

FIG. 7A

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L fragment	Cons-p9	L1*(A)	L2(A)	L6(A)	L8(A)	L9(A)	L3(B)	L7*(B)	L10(B)	L4(C)	Cons-D	Cons-F
E2	E	T	T	T	.	E,T	E,T
384	H	V	V	V	.	H,Y	H,Y
386	T	S	S	S	.	T,S	T,S
388	R	G	G	G	.	G	R,G
390	V	A	V	.	V
391	A	V	.	.	V	V	V	A,V
392	H	R	R	R	R	.	H
394	S	P	H	.	.	S
405	Q	H	H	H	.	H	Q,H
434	F	L	L	L	L	L	F,L
438	A	T	T	T	T	T	A,T
444	S	P	.	S
450	S	S
458	A	.	.	.	N	.	V	V	V	.	A,V	A,V
466	Y	H	Y
474	K	E	E	E	E	E	K,E
476	V	I	I	I	I	I	V,I
496	V	A	.	A	.	.	.	V
524	V	V
536	I	.	M	V	.	.	.	I
580	L	V	L
622	Q	.	.	.	P	Q
673	A	V	.	.	.	A
783	A	A

p7

FIG. 7B

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	L fragment	Cons-p9	L1* (A)	L2(A)	L6(A)	L8(A)	L9(A)	L3(B)	L7* (B)	L10(B)	L4(C)	Cons-D	Cons-F
NS2	820	G	S	.	.	.	G
	857	M	I	M
	927	K	R	.	.	.	K
	934	V	I	I	.	I	I	V
	937	A	.	.	V	.	.	D	.	D	.	D	A,D
	978	A	P
	1028	P	.	.	.	S	A
	1031	A	I	.	I	.	I	V,I
	1043	V	.	.	I	.	.	H	H	H	.	Q,H	Q,H
	1067	Q	I
NS3	1097	I	X	G
	1188	G	R	S
	1215	S	.	S	T	F
	1223	F	A
	1226	A	V	.	.	A
	1339	A	V	K
	1399	K	N	T
	1503	T	S	.	S	.	.	Y
	1528	Y	T
	1535	T	A	L
	1662	L	.	P
NS4A													

FIG. 7C

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	L fragment	Cons-p9	L1*(A)	L2(A)	L6(A)	L8(A)	L9(A)	L3(B)	L7*(B)	L10(B)	L4(C)	Cons-D	Cons-F
NS4B	1753	K	.	R	K
	1805	H	.	.	N	.	.	N	.	N	N	N	H,N
	1949	S	P	P	.	S
	2105	M	V	I	.	I	.	.	M
	2136	K	R	R	.	K
NS5A	2146	T	A	A	A	.	T,A	T,A
	2226	L	P	L
	2259	L	F	.	.	.	E,D	E,D
	2262	E	D	D	D	.	.	V
	2334	V	I	.	.	.	L,Q	L,Q
	2371	L	Q	Q	Q	H	.	Y
	2385	Y	N
	2692	N	S	.	.	.	A
	2757	A	C
	2785	C	.	R	I
NS5B	2824	I	.	V	A
	2861	A	V
	S fragment		S5	S9	S2	S3	S7	S8	S10	S4	S6	.	G
	2968	G	S	S	.	I	.	S
	2975	S	G	G	G	G	.	D
	2978	D	S
	2999	S	.	F	F	F

FIG. 7D

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aa \ nt	L1 (A)	L2 (A)	L6 (A)	L8 (A)	L9 (A)	L3 (B)	L7 (B)	L10 (B)	L4 (C)	HC-J4/91	HC-J4/83
L1 (A)		0.56	0.60	0.36	0.33	1.50	1.53	1.46	0.95	0.83	1.79
L2 (A)	0.59		0.55	0.35	0.50	1.49	1.51	1.45	0.98	0.82	1.77
L6 (A)	0.52	0.42		0.31	0.55	1.33	1.38	1.29	0.80	0.68	1.58
L8 (A)	0.42	0.38	0.31		0.31	1.32	1.34	1.28	0.79	0.65	1.62
L9 (A)	0.35	0.52	0.45	0.35		1.42	1.42	1.38	0.91	0.75	1.66
L3 (B)	1.47	1.43	1.15	1.33	1.36		0.61	0.30	1.43	0.90	1.51
L7 (B)	1.36	1.33	1.05	1.22	1.22	0.66		0.57	1.47	0.95	1.54
L10 (B)	1.36	1.33	0.59	1.22	1.26	0.31	0.56		1.37	0.85	1.42
L4 (C)	0.77	0.80	0.59	0.63	1.26	1.12	1.08	1.01		0.76	1.73
HC-J4/91	0.94	0.91	0.63	0.80	0.87	0.77	0.73	0.66	0.52		1.22
HC-J4/83	1.96	1.89	1.68	1.85	1.82	1.75	1.61	1.61	1.71	1.40	

FIG. 8

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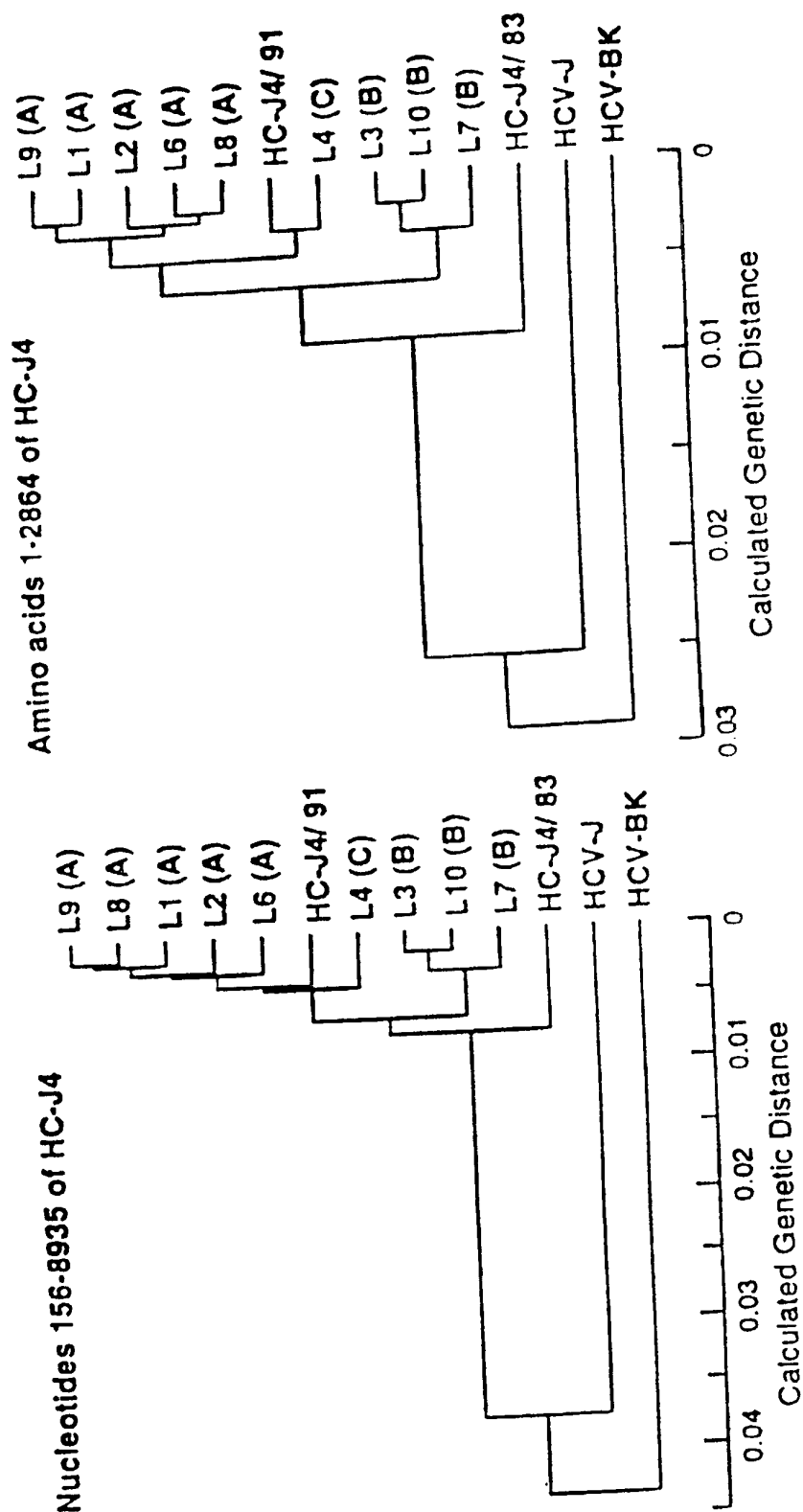


FIG. 9

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	468		486
	GWGPIT	YTKPNSS	DORPYC

	E.....

	G.....

	E.....
	E.....
	A.....

	E.....
	E.....
	H.E.....
	G.D.L.....
	E.....
	E...P.....
	E...P.....
	ESG.R.....
	E.D.P.....
			HVR2
HC-J4L6 (A) :	AGVDG	ETHHTGRVAGHTTSGFTSLESSGAS	QKIQL
HC-J4L2 (A) :
HC-J4/91-20 :	R.....
HC-J4L1 (A) :	V.....
HC-J4L8 (A) :	V.....
HC-J4L9 (A) :	V.....
HC-J4/91-21 :	V.....
HC-J4L4 (C) :	V.R.....
HC-J4/91-23 :	V.R.....
HC-J4/91-22 :	V.R.....
HC-J4L7 (B) :	T.Y.S.G.....	R.....P.....
HC-J4L10 (B) :	T.....	T.Y.S.G.A.....	R.....
HC-J4L3 (B) :	T.....	T.Y.S.G.....	R.....
HC-J4/91-26 :	T.....	T.Y.S.G.....	R.....
HC-J4/91-25 :	A.Y.S.G.....	R.....
HC-J4/91-24 :	A.Y.S.G.....	R.....
HC-J4/91 :	A.Y.S.G.....	R.....
HC-J4/91-27 :	K.Y.S.G.A.S.....	R.....P.....
HC-J4/83 :	Y.S.G.A.S.....	TLA.....P.....
			HVR1

FIG. 10

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90
 1
 180
 270
 341

3' Untranslated Region

IC-J4 :TTAGTATGAG TGTCGTGCAG CCTCCAGGAC CCCCCCTCCC GGGAGAGCCA TAGTGGTCTG CGGAACCGGT GAGTACACCG GAATTGCCAG
 CV-J4L6S :GCCAGCCCCC GATTGGGGC GACACTCCAC CATAGATCAC TCCCCGTGTA GGAAGCGTCT AGCCATGGCG
 CV-H77C :TGA.....TGA.....GA.....GA.....

91
 181
 271

IC-J4 :TAGTATGAG TGTCGTGCAG CCTCCAGGAC CCCCCCTCCC GGGAGAGCCA TAGTGGTCTG CGGAACCGGT GAGTACACCG GAATTGCCAG
 CV-J4L6S :GCCAGCCCCC GATTGGGGC GACACTCCAC CATAGATCAC TCCCCGTGTA GGAAGCGTCT AGCCATGGCG
 CV-H77C :TGA.....TGA.....GA.....GA.....

9372
 9513

3' variable region

poly U-UC region

3' variable region

9514
 9595

3' conserved region (Cont.)

HC-J4 :TGAACGGGGA GCTAACCACT CCAGGCCAAT AGGCCTT--C CTG poly (U-UC)n
 CV-J4L6S :.....T..A..A..TT. ... poly (U-UC)81
 CV-H77C :.....G..TT.G..G...A....G.C.TCT..A..A..TT. ... poly (U-UC)81
 Bfr 1

9514
 9595

3' conserved region (Cont.)

HC-J4 :CCCTAGTCAC GGCTAGCTGT GAAAGGTCCG TGAGCCGCAT GACTGCAGAG AGTGTGATA CTGGCCCTC TGCAGATCAT GT
 CV-J4L6S :.....
 CV-H77C :.....

FIG.11

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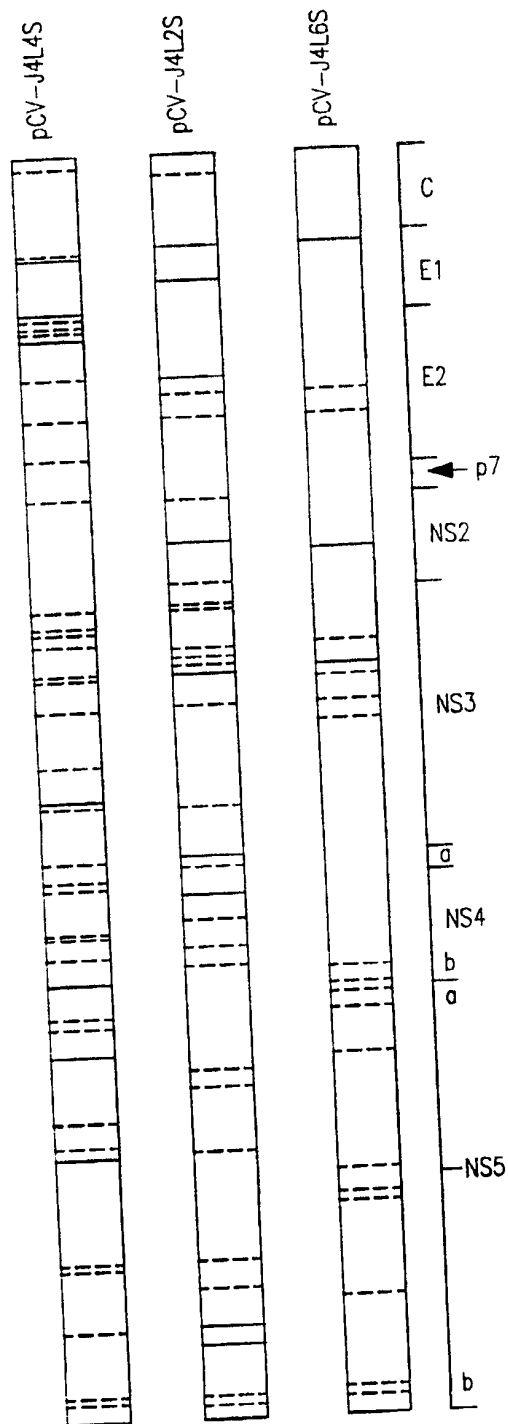


FIG. 12

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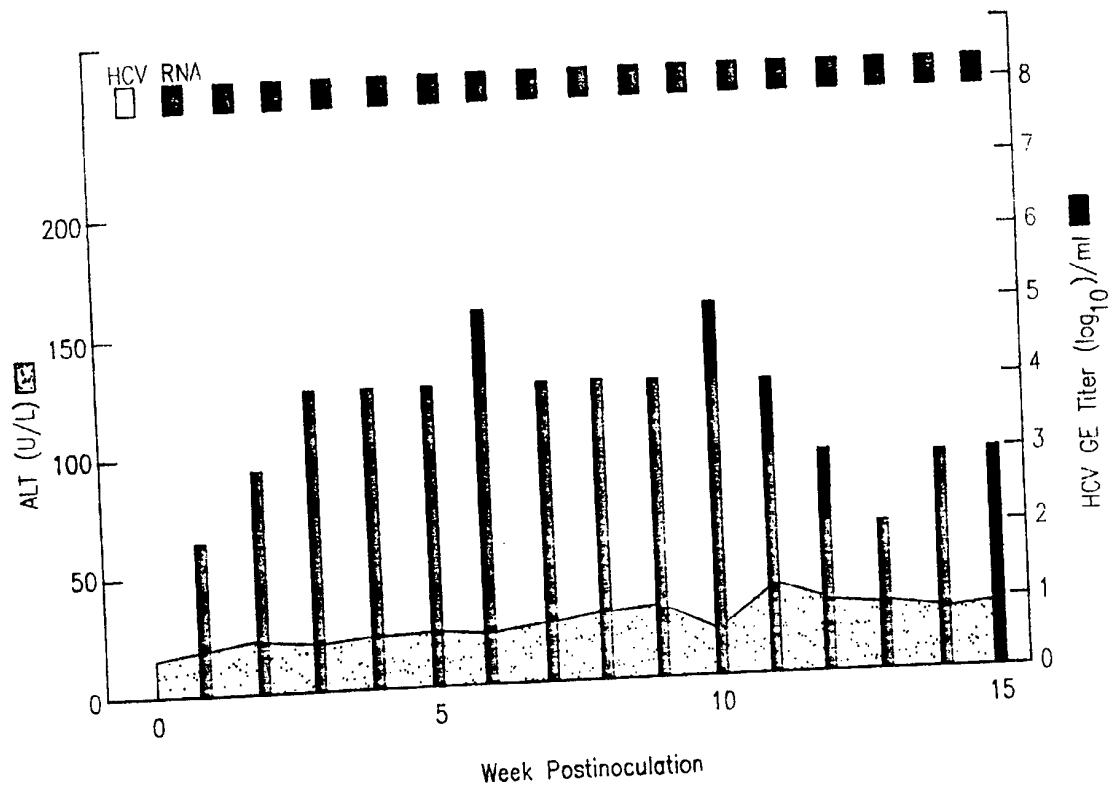


FIG. 13

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HC-J4

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
GCCAGCCCCC	TGATGGGGGC	GACACTCCAC	CATGAATCAC	TCCCCCTGIGA	50
GGAACACTG	TCTTCACGCA	GAAAGCGTCT	AGCCATGGCG	TTAGTATGAG	100
TGTGCTGCAG	CCTCCAGGAC	CCCCCTCCC	GGGAGAGCCA	TAGTGGTCTG	150
CGGAACCGGT	GAGTACACCG	GAATTGCCAG	GACGACCGGG	TCCTTTCTTG	200
GATCAACCCG	CTCAATGCCT	GGAGATTTGG	GCGTGCCCCC	GCGAGACTGC	250
TAGCCGAGTA	GTGTTGGGTC	GCGAAAGGCC	TTGTGGTACT	GCCTGATAGG	300
GTGCTTGCGA	GTGCCCCGGG	AGGTCTCGTA	GACCGTGCAC	CATGAGCACG	350
AATCCTAAAC	CTCAAAGAAA	AACCAAACGT	AACACCAACC	GCCGCCACAA	400
GGACGTCAAG	TTCCCCGGCG	GTTGGTCAGAT	CGTTGGTGGG	GTTTACCTGT	450
TGCCGCGCAG	GGGCCCCAGG	TTGGGTGTGC	GCGCGACTAG	GAAGGCTTCC	500
GAGCGGTGCG	AACCTCGTGG	AAGGCGACAA	CCTATCCCAA	AGGCTCGCCG	550
ACCCGAGGGC	AGGGCCTGGG	CTCAGCCCGG	GTACCCCTTG	CCCCCTCTATG	600
GCAATGAGGG	CCTGGGGTGG	GCAGGATGGC	TCCTGTACCC	CCGCGGCTCC	650
CGGCCTAGTT	GGGGCCCCAC	GGACCCCGGG	CGTAGGTGCG	GTAACCTGGG	700
TAAGGTCAATC	GATACCCCTTA	CATGCGGCTT	CGCCGATCTC	ATGGGGTACA	750
TTCCGCTCGT	CGGCGCCCCC	CTAGGGGGGG	CTGCCAGGGC	CTTGGCACAC	800
GGTGTCCGGG	TTCTGGAGGA	CGGCGTGAAC	TATGCAACAG	GGAACCTGCC	850
CGGTTGCTCT	TTCTCTATCT	TCCTCTTTGG	TCTGCTGTCC	TGTTTGACCA	900
TCCCAGCTTC	CGCTTATGAA	GTGCGCAACG	TGTCCGGGAT	ATAACCATGTC	950
ACGAACGACT	GCTCCAACTC	AAGCATTTGT	TATGAGGCAG	CGGACGTGAT	1000
CATGCATACT	CCCGGGTGGG	TGCCCCGTGT	TCAGGAGGGT	AACAGCTCCC	1050
GTGCTGGGGT	AGCGCTCACT	CCCACGCTCG	CGGCCAGGAA	TGCCAGCGTC	1100
CCCACTACGA	CAATACGACG	CCACGTGCGAC	TTGCTCGTTG	GGACGGCTGC	1150
TTTCTGCTCC	GCTATGTACG	TGGGGGATCT	CTGCGGATCT	ATTTTCTCTG	1200
TCTCCAGCT	GTTCACCTTC	TGCGCTCGCC	GGCATGAGAC	AGTGCAGGAC	1250
TGCAACTGCT	CAATCTATCC	CGGCCAATGTA	TCAGGTCACC	GCATGGCTTG	1300
GGATATGATG	ATGAACTGGT	CACCTACAAC	AGCCCTAGTG	GTGTGCGAGT	1350
TGCTCCGGAT	CCCACAAGCT	GTGTTGGACA	TGGTGGGGGG	GGGCCACTGG	1400
GGAGTCCCTG	CGGGCCCTTG	CTACTATTCC	ATGGTATGGG	ACTGGGCTAA	1450
GGTTCTGATT	GTGGCGCTAC	TCTTTTGCCG	CGTTGACGGG	GAGACCCACA	1500
CGACGGGGAG	GGTGGCCGGC	CACACCAOCT	CCGGGTTCAC	GTCCCTTTTC	1550
TCATCTGGGG	CGTCTCAGAA	AATCCAGCTT	GTGAATACCA	ACGGCAGCTG	1600
GCACATCAAC	AGGACTGCCC	TAAATTGCAA	TGACTCCCTC	CAAACCTGGT	1650
TCTTTGCCCG	GCTGTTTTAC	GCACACAAGT	TCAACTCGTC	CGGGTGCCCC	1700
GAGCGCATGG	CCAGCTGCGG	CCCCATTGAC	TGGTTCGCCC	AGGGGTGGGG	1750
CCCCATCACC	TATACTAAGC	CTAACAGCTC	GGATCAGAGG	CCTTATTTGCT	1800
GGCATTACGC	GOCTCGACCG	TGTTGGTGTG	TACCCGCGTC	GCAGGTGTGT	1850
GGTCCAGTGT	ATTGTTTCAC	CCCAAGCCCT	GTGTGGTGGG	GGACCACCGA	1900

FIG. 14A

SUBSTITUTE SHEET (RULE 26)

HC-J4

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
TCGTTCCGGT	GTCCCTACGT	ATAGCTGGGG	GGAGAATGAG	ACAGACGTGA	1950
TGCTCCTCAA	CAACACGGGT	CCGCCACAAG	GCAACTGGTT	CGGCTGTACA	2000
TGGATGAATA	GTACTGGGTT	CACTAAGACG	TGCGGAGGTC	CCCCGTGTAA	2050
CATCGGGGGG	GTCCGTAAAC	GCACCTTGAT	CTGCCCCACG	GA CTGCTTCC	2100
GGAAGCACCC	CGAGGCTACT	TACACAAAAT	GTGGCTGGGG	GCCCCTGGTG	2150
ACACCTAGGT	GCCTAGTAGA	CTACCCATAC	AGGCTTTGGC	ACTACCCCTG	2200
CACTCTCAAT	TTTTCCATCT	TTAAGGTTAG	GATGTATGTG	GGGGGGGTGG	2250
AGCACAGGCT	CAATGCCGCA	TGCAATTGGA	CTCGAGGAGA	GCGCTGTAAAC	2300
TTGGAGGACA	GGGATAGGTC	AGAACTCAGC	CCGCTGCTGC	TGCTCTACAAC	2350
AGAGTGGCAG	ATACTGCCCC	GTGCTTTTAC	CACCCCTACG	GCTTTATCCA	2400
CTGGTTTGAT	CCATCTCCAT	CAGAACATCG	TGGACGTGCA	ATACCTGTAC	2450
GGTGTAGGGT	CAGCGTTTGT	CTCCTTTTGA	ATCAAATGGG	AGTACATCCT	2500
GTTCCTTTTC	CTTCTCCTGG	CAGACGCGCG	CGTGTGTGOC	TGCTGTGTGA	2550
TGATGCTGCT	GATAGCCCAG	GCTGAGGCCG	CCTTAGAGAA	CTTGGTGGTC	2600
CTCAATGCGG	CGTCCGTGGC	CGGAGCGCAT	GGTATTCTCT	CCTTTCTTGT	2650
GTTCCTTCTG	GCCCCTGGT	ACATTAAAGG	CAGGCTGGCT	CCTGGGGGGG	2700
CGTATGCTTT	TTATGGGGTA	TGGCCGCTGC	TCCCTGCTCT	ACTGGGGTTA	2750
CCACCACGAG	CTTACGCCCT	GGACCGGGAG	ATGGCTGCAT	CGTCCGGGGG	2800
TGCGGTTCTT	GTAGGTCTGG	TATTCTTTGAC	CTTGTACCCA	TACTACAAAG	2850
TGTTTCTCAC	TAGGCTCATA	TGGTGGTTAC	AATACTTTAT	CACCAGAGCC	2900
GAGGCGCACA	TGCAAGTGTG	GGTCCCCCCC	CTCAACGTTT	GGGGAGGCCG	2950
CGATGCCATC	ATCCTCCTCA	CGTGTGCGGT	TCATCCAGAG	TTAATTTTTG	3000
ACATCACCAA	ACTCCTGCTC	GCCATACTCG	GCCCCGTCAT	GGTGTCCAG	3050
GCTGGCATAA	CGAGAGTGGC	GTACTTCGTG	CGCGCTCAAG	GGCTCATTCG	3100
TGCATGCATG	TTAGTGGGAA	AAGTCCCGCG	GGGTCAATTAT	GTCCAAATGG	3150
TCTTCATGAA	GCTGGGGCGG	CTGACAGGTA	CGTACGTTTA	TAACCATCTT	3200
ACCCCACTGC	GGGACTGGGC	CCACGGGGGC	CTACGAGACC	TTGCGGTGGC	3250
GGTAGAGCCC	GTGCTCTTCT	CCGCCATGGA	GACCAAGGTC	ATCACCTGGG	3300
GAGCAGACAC	CGCTGCGTGT	GGGGACATCA	TCTTGGGTCT	ACCCGCTTCC	3350
GCCCGAAGGG	GGAAGGAGAT	ATTTTTTGGG	CCGGCTGATA	GTCTCGAAGG	3400
GCAAGGGTGG	CGACTCCCTG	CGCCCATCAC	GGCCTACTCC	CAACAACGCG	3450
GGGGCGTACT	TGGTTGCATC	ATCACTAGCC	TCACAGGCCG	GGACAAGAAC	3500
CAGGTGGAAG	GGGAGGTTCA	AGTGGTTTCT	ACCGCAACAC	AATCTTTTCT	3550
GGCGACCTGC	ATCAACGGCG	TGTGCTGGAC	TGTCTTACCAT	GGCGCTGGCT	3600
CGAAGACCC	AGCCGGTCCA	AAAGGTCCAA	TCACCCAAAT	GTACACCAAT	3650
GTAGACCTGG	ACCTCGTCCG	CTGGCAGGGG	CCCCCGGGGG	CGCGCTCCAT	3700
GACACCATGC	AGCTGTGGCA	GCTCGGACCT	TTACTTGGTC	ACGAGACATG	3750
CTGATGTCAT	TCCGGTGGCG	CGGCGAGGCG	ACAGCAGGGG	AAGTCTACTC	3800

FIG. 14B

HC-J4

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
TCCCCAGGC	CCGTCCTCTA	CCTGAAAGGC	TCCTGGGGTG	GTCCATTGCT	3850
TTGCOOCTTG	GGGCAOGTG	TGGGCGTCTT	CCGGGCTGCT	GIGTGCAACC	3900
GGGGGGTGGC	GAAGGCGGTG	GACTTCATAC	CCGTTGAGTC	TATGGAAACT	3950
ACCATGCGGT	CTCCGGTCTT	CACAGACAAC	TCAACCCCCC	CGGCTGTACC	4000
GCAGACATTC	CAAGTGGCAC	ATCTGCACGC	TCCTACTGGC	AGCGGCAAGA	4050
GCACCAAAGT	GCCGGCTGGG	TATGCAGGCC	AAGGGTACAA	GGTGCTGGTC	4100
CTGAACCCGT	CCGTTGCCCC	CACCTTAGGG	TTTGGGGGGT	ATATGTCCAA	4150
GGCACACGGT	ATCGACCCCTA	ACATCAGAAC	TGGGGTAAGG	ACCATTACCA	4200
CGGGCGGCTC	CATTACGTAC	TCCACCTATG	GCAAGTTCTT	TGCCGACGGT	4250
GGCTGTTCTG	GGGGCGCCTA	TGACATCATA	ATATGTGATG	AGTGGCCTC	4300
AAC TGACTCG	ACTACCATCT	TGGGCATGGG	CACAGTCTTG	GACCAAGGGG	4350
AGACGGCTGG	AGCGCGGCTC	GTGGTGCTCG	CCACCGCTAC	ACCTCCGGGA	4400
TGGGTTACCG	TGCCACAACC	CAATATCGAG	GAAATAGGCC	TGTCCAACAA	4450
TGGAGAGATC	CCCTTCTATG	GCAAAGOCAT	CCCCATTGAG	GCCATCAAGG	4500
GGGGGAGGCA	TCTCATTTTC	TGCCATTCCA	AGAAGAAATG	TGACGAGCTC	4550
GCCGCAAAGC	TGACAGGCCT	CGGACTGAAC	GCTGTAGCAT	ATTACCGGGG	4600
CCTTGATGTG	TCCGTTCATAC	CGCCTATCGG	AGACGTGGTT	GTGGTGGCAA	4650
CAGACGCTCT	AATGACGGGT	TTCACCGGCG	ATTTTGACTC	AGTGATCGAC	4700
TGCAATACAT	GTGTACCCCA	GACAGTCGAC	TTCAGCTTGG	ATCCACCTTT	4750
CACCAATTGAG	ACGACGACCG	TGCCCCAAGA	CGCGGTGTG	CGCTCCCAAC	4800
GGCGAGGTAG	AAC TGGCAGG	GGTAGGAGTG	GCATCTACAG	GTTTGIGACT	4850
CCAGGAGAAC	GGCCCTCGGG	CATGTTGGAT	TCTTCGGTCC	TGTGTGAGTG	4900
CTATGACGGG	GGCTGTGCTT	GGTATGAGCT	CACGCCCCGT	GAGACCTCGG	4950
TTAGGTTGGG	GGCTTACCTA	AATACACCAG	GGTTGCCCCG	CTGCCAGGAC	5000
CATCTGGAGT	TCTGGGAGAG	CGTCTTCACA	GGCTTCACCC	ACATAGATGC	5050
CCACTTCTCTG	TCCAGACTA	AACAGGCAGG	AGACAACTTT	CCTTACCTGG	5100
TGGCATATCA	AGCTACAGTG	TGCGCCAGGG	CTCAAGCTCC	ACCTCCATCG	5150
TGGGACCAAA	TGTGGAAGTG	TCTCATACCG	CTGAAACCTA	CACTGCACGG	5200
GCCAACACCC	CTGCTGTATA	GGCTAGGAGC	CGTCCAAAAT	GAGGTATCC	5250
TCACACACCC	CATAACTAAA	TACATCATGG	CATGCATGTC	GGCTGACCTG	5300
GAGGTGCTCA	CTAGCACCTG	GGTGCTGGTA	GGCGGAGTCC	TTCAGCTTTT	5350
GGCCGCATAC	TGCTTGACGA	CAGGCAGTGT	GGTCATTGIG	GGCAGGATCA	5400
TCTTGTCGGG	GAAGCCAGCT	GTGCTTCCCG	ACAGGGGAAGT	CCTCTACCAG	5450
GAGTTCGATG	AGATGGAAGA	GTGTGCCTCA	CAACTTCCTT	ACATCGAGCA	5500
GGGAATGCAG	CTCGCCGAGC	AATTCAAGCA	AAAGGCGCTC	GGGTTGTTGC	5550
AAACGGCCAC	CAAGCAAGCG	GAGGCTGCTG	CTCCCGTGGT	GGAGTCCAG	5600
TGGCGAGCCC	TTGAGACCTT	CTGGGCGAAG	CACATGTGGA	ATTTCATCAG	5650
CGGAATACAG	TACCTAGCAG	GCTTATCCAC	TCTGCCTGGA	AACCCCGCGA	5700

FIG. 14C

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HC-J4

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
TAGCATCATT	GATGGCATT	ACAGCTTCTA	TCTAGCCC	GCTCACCACC	5750
CAAAACACCC	TCCTGTTTAA	CATCTTGGGG	GGATGGGTGG	CTGCCCCAACT	5800
CGCTCCTCCC	AGCGCTGCGT	CAGCTTTTGGT	GGCGCCCGGC	ATCGCCCGGAG	5850
CGGCTGTGGG	CAGCATAGGC	CTTGGGAAGG	TGCTCGTGGG	CATCTTGGCG	5900
GGCTATGGGG	CAGGGGTAGC	CGGCGCACTC	GTGGCCTTTA	AGGTTCATGAG	5950
CGGCGAGGTG	CCCTCCACCG	AGGACCTGGT	CAACTTACTC	CCTGCCATCC	6000
TCTCTCCTGG	TGCCCIGGTC	GTCGCGGTGG	TGTGCGCAGC	AATACTGGGT	6050
CGGCACGTGG	GCCCCGGAGA	GGGGGCTGTG	CAGTGGATGA	ACCGGCTGAT	6100
AGCGTTGGCT	TGCGGGGGTA	ACCACGTCCT	CCCTACGCAC	TATGTGGCTG	6150
AGAGCGACGC	TGCAGCACGT	GTCACTCAGA	TCTCTCTAG	CCTTACCATC	6200
ACTCAACTGC	TGAAGCGGCT	CCACCACTGG	ATTAAATGAGG	ACTGCTCTAC	6250
GCCATGCTCC	GGCTCGTGGC	TAAGGGATGT	TGGGATTGG	ATATGCCACGG	6300
TGTTGACTGA	CTTCAAGACC	TGGCTCCAGT	CCAAACTCCT	GCCGCGGTGA	6350
CCGGGAGTCC	CTTTCTGTG	ATGCCAACGC	GGGTACAAGG	GAGTCTGGCG	6400
GGGGGACGGC	ATCATGCAAA	CCACCTGCCC	ATGCGGAGCA	CAGATGGCCG	6450
GACATGTCAA	AAACGGTTC	ATGAGGATCG	TAGGGCTAG	AACCTGCAGC	6500
AACACGTGGC	ACGGAACGTT	CCCCATCAAC	GCATACACCA	CGGGACCTTG	6550
CACACCTCC	CCGGCGCCCA	ACTATTCCAG	GGGCTATGG	CGGGTGGCTG	6600
CTGAGGAGTA	CGTGGAGGTT	ACCGGTGTGG	GGGATTTCOA	CTACGTGACG	6650
GGCATGACCA	CTGACAACGT	AAAGTGCCCA	TGCCAGGTTT	CGGCCCCCGA	6700
ATTCTTCACG	GAGGTGGATG	GAGTGCGGTT	GCACAGGTAC	GCTCCGGCGT	6750
GCAAACCTCT	TCTACGGGAG	GACGTACAGT	TCCAGGTCCG	GCTCAACCAA	6800
TACTTGGTGG	GGTCCAGCT	CCCATGCGAG	CCCGAACCGG	ACGTAAACAGT	6850
GCTTACTTCC	ATGCTCACCG	ATCCCTCCCA	CATTACAGCA	GAGACGGCTA	6900
AGCGTAGGCT	GGCTAGAGGG	TCTCCCCCTT	CTTTAGCCAG	CTCATCAGCT	6950
AGCCAGTTGT	CTGCGCCTTC	TTTGAAGGCG	ACATGCACTA	CCCACCATGA	7000
CTCCCCGGAC	GCTGACCTCA	TGAGGGCCAA	CCTCTTGTGG	CGGCAGGAGA	7050
TGGGCGGAAA	CATCACTGGC	GTGGAGTCAG	AGAATAAGGT	AGTAATTCTG	7100
GACTCTTTGG	AACCGCTTCA	CGCGGAGGGG	GATGAGAGGG	AGATATCCGT	7150
CGCGGCGGAG	ATCCTGCGAA	AATCCAGGAA	GTCCCCCTCA	GCGTTGCCCA	7200
TATGGGCACG	CCCGGACTAC	AATCCTCCAC	TGCTAGAGTC	CTGGAAGGAC	7250
CCGGACTACG	TCCCTCCGGT	GGTACACGGA	TGCCCATTTG	CACCTACCAA	7300
GGCTCCTCCA	ATACCACCTC	CACGGAGAAA	GAGGACGGTT	GTCTGACAG	7350
AATCCAATGT	GTCTCTGCG	TTGGCGGAGC	TGCGCACTAA	GACCTTCGGT	7400
AGCTCCGGAT	CGTCCGGCGT	TGATAGCGGC	ACGGCGACCG	CCCTTCCTGA	7450
CCTGGCCTCC	GACGACGGTG	ACAAAGGATC	CGACGTGTAG	TGCTACTCCT	7500
CCATGCCCCC	CCTTGAAGGG	GAGCCGGGGG	ACCCCGATCT	CAGCGACGGG	7550
TCTTGGTCTA	CCGTGAGTGA	GGAGGCTAGT	GAGSATGTGG	TCTGCTGCTC	7600

FIG. 14D

SUBSTITUTE SHEET (RULE 26)

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HC-J4

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
AATGTCTTAT	ACGTGGACAG	GCGCCCTGAT	CACGCCATGC	GCTGCGGAGG	7650
AAAGTAAGCT	GCCCATCAAC	CCGTTGAGCA	ACTCTTTTGT	GCGTCAACCAC	7700
AACATGGTCT	ACGCCACAAC	ATCCCGCAGC	GCAAGCCTCC	GGCAGAAGAA	7750
GGTCACTTTT	GACAGATTGC	AAGTCTTGGA	TGATCATTAC	CGGGACGTAC	7800
TCAAGGAGAT	GAAGGCGAAG	GCGTCCACAG	TTAAGGCTAA	GCTTCTATCT	7850
ATAGAGGAGG	CCTGCAAGCT	GACGCCCCCA	CATTCCGSCA	AATCCAAATT	7900
TGGCTATGGG	GCAAAGGACG	TCCGGAACCT	ATCCAGCAGG	GCCGTTAACC	7950
ACATCCGCTC	CGTGTGGGAG	GACTTGTCTG	AAGACTCTGA	AACAACAATT	8000
GACACCACCA	TCATGGCAAA	AAGTGAGGTT	TTCTGGGTCC	AACCAGAGAA	8050
GGGAGGCCGC	AAGCCAGCTC	GCCTTATCGT	ATTCCCAGAC	CTGGGAGTTC	8100
GTGTATGGCA	GAAGATGGCC	CTTTACGACG	TGGTCTCCAC	CCTTCCCTCAG	8150
GCCGTGATGG	GCTCCTCATA	CGGATTTTCAA	TACTCCCCCA	AGCAGCGGGT	8200
CGAGTTCTTG	GTGAATACCT	GGAAATCAAA	GAAATGCCCT	ATGGGCTTCT	8250
CATATGACAC	CCGCTGTTTT	GACTCAACGG	TCACTGAGAG	TGACATTGGT	8300
GTTGAGGAGT	CAATTTCACCA	ATGTTGTGAC	TTGGCCCCCG	AGGCCAGACA	8350
GGCCATAAGG	TGGCTCACAG	AGCGGCTTTA	CATCGGGGGT	CCCCTGACTA	8400
ACTCAAAAGG	GCAGAACTGC	GGTTATCGCC	GGTGCCCGGC	AAGTGGCGTG	8450
CTGACGACTA	GCTGCGGTAA	TACCTTCACA	TGTTACTTGA	AGGCCACTGC	8500
AGCCTGTGGA	GCTGCAAAGC	TCCAGGACTG	CACGATGCTC	GTGAACGGAG	8550
ACGACCTTGT	CGTTATCTGT	GAAAGCGCGG	GAACCCAGGA	GGATGCGGCG	8600
GCCCTACGAG	CCTTCACGGA	GGCTATGACT	AGGTATTCCG	CCCCCCCCCG	8650
GGATCCGCCC	CAACCAGAAT	ACGACCTGGA	GCTGATAACA	TCATGTTTCT	8700
CCAATGTGTC	AGTCGCGCAC	GATGCATCTG	GCAAAAGGGT	ATACTACCTC	8750
ACCCGTGACC	CCACCACCCC	CCTTGCAACG	GCTGCGTGGG	AGACAGCTAG	8800
ACACACTCCA	ATCAACTCTT	GGCTAGGCAA	TATCATCATG	TATGCGCCCA	8850
CCCTATGGGC	AAGGATGATT	CTGATGACTC	ACTTTTCTTC	CATCCTTCTA	8900
GCTCAAGAGC	AACTTGAAAA	AGCCCTGGAT	TGTCAGATCT	ACGGGGCTTG	8950
CTACTCCATT	GAGCCACTTG	ACCTACCTCA	GATCATTGAA	CGACTCCATG	9000
GTCTTAGCGC	ATTTACACTC	CACAGTTACT	CTCCAGGTGA	GATCAATAGG	9050
GTGGCTTCAT	GCCTCAGGAA	ACTTGGGGTA	CCACCCCTGC	GAACCTGGAG	9100
ACATCGGGCC	AGAAGTGTCC	GCGCTAAGCT	ACTGTCCCAG	GGGGGGAGGG	9150
CCGCCACTTG	TGGCAGATAC	CTCTTTAACT	GGGCAGTAAG	GACCAAGCTT	9200
AAACTCACTC	CAATCCCGGC	CGCGTCCCAG	CTGGACTTGT	CTGGCTGGTT	9250
CGTCGCTGGT	TACAGCGGGG	GAGACATATA	TCACAGCCTG	TCTCGTGGCC	9300
GACCCCGCTG	GTTTCCGTTG	TGCTTACTCC	TACTTTCTGT	AGGGGTAGGC	9350
ATTTACCTGC	TCCCCAACCG	ATGAACGGGG	AGCTAACCAC	TCCAGGCCTT	9400
AAGCCATTTC	CTGTTTTTTT	TTTTTTTTTT	TTTTTTTTTT	TCTTTTTTTT	9450
TTTCTTCTCT	TTCCTTCTTT	TTTTCCTTTC	TTTTTCCCTT	CTTTAATGGT	9500

FIG. 14E

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10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
GGCTCCATCT	TAGCCCTAGT	CACGGCTAGC	TGIGAAAGGT	CCGIGAGCCG	9550
CATGACTGCA	GAGAGTGCTG	ATACTGGCCT	CTCTGCAGAT	CATGT	9595

FIG. 14F

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10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
MSINPKPQRK	TKRNINRRPQ	DVKFPGGGQI	VGGVYLLPRR	GPRLGVRATR	50
KASERSQPRG	RRQPIPKARR	PEGRAWAQPG	YFWPLYGNEG	LGWAGWLLSP	100
RGSRPSWGPT	DPRRRSRNLG	KVIDTILTCGF	ADLMGYIPLV	GAPLGGAARA	150
LAHGVRVLED	GVNYATGNLP	GCSFSIFLLA	LLSCLTIPAS	AYEVRNVSGI	200
YHVINDCSNS	SIVYEAADVI	MHTPGCVPCV	QEGNSSROW	ALTPILAARN	250
ASVPTTITRR	HVDLLVGIAA	FCSAMYVGDL	CGSIFLVSQL	FTFSPPRHET	300
VQDNCSTYP	GHVSGHRMAW	DMMNWSPTT	ALVVSQLLRI	PQAVVDMVAG	350
AHWGLAGLA	YYSMVGNWAK	VLIVALLFAG	VDGETHTTGR	VAGHTTSGFT	400
SLFSSGASQK	IQLVNINGSW	HINRTALNCH	DSLQGTGFFAA	LFYAHKFNSS	450
GCPERMASCR	PIDWFAQGWG	PITYTKFNSS	DQRPYCWHYA	PRPGVVPAS	500
QVCGPVYCF	PSPVAVGTTD	RSQVPTYSWG	ENETDMLLN	NIRPPQGNWF	550
GCTWMNSTGF	TKTCGGPPCN	IGGVGNRILI	CPTDCFRKHP	EATYTKCGSG	600
PWLTPRCLVD	YPYRLWHYPC	TINFESIFKVR	MYVGGVEHRL	NAACNWIRGE	650
RCNLEDRDRS	ELSPLLLSTT	EWQILPCAFT	TLPALSTGLI	HLHQNIVDVQ	700
YLYGVGSAFV	SFAIKWEYIL	LLFLILLADAR	VCACLIWMLL	IAQAEAALEN	750
LWVLNAAVA	GAHGILSFLV	FFCAAWYIKG	RLAPGAAYAF	YGVWPLLLLL	800
LALPPRAYAL	DREMAASCGG	AVLVGLVFLT	LSPYYKVFLT	RLIWNLOFYI	850
TRAEAHMQW	VPPLNVRGGR	DAIILLTCAV	HPELIFDITK	LLLATILGPLM	900
VLQAGITRVP	YFVRAQGLIR	ACMLVRKVAG	GHYVQMVFMK	LGALTGTIVY	950
NHLLPLRDWA	HAGLRDLAVA	VEPVVFSAME	TKVTTWGADT	AACGDIILGL	1000
FVSARRKEI	FLGPADSLEG	QGWRLAPIT	AYSQOTRGVL	GCIITSLTGR	1050
DKNQVEGEVQ	VVSTATQSFL	ATCINGVCWT	VYHGAGSKIL	AGPKGPITQM	1100
YINVDLDELVG	WQAPPGARSM	TPCSGSSDL	YLVTRHADVI	PVRRRGDSRG	1150
SLLSRPVSY	LKGSSGGPLL	CPSGHVGVF	RAAVCIRGVA	KAVDFIPVES	1200
METIMRSEVF	TDNSTPPAVP	QTFQVAHLHA	PTGSGKSTKV	PAAYAAQGYK	1250
VLVLNPSVAA	TLGFGAYMSK	AHGIDENIRT	GVRTTTTGGG	ITYSTYKFL	1300
ADGGCSGGAY	DIICDECHS	TDSTTILGIG	TVLDQAETAG	ARLVVLATAT	1350
PPGSVIVPHP	NIEETGLSNN	GEIPFYGKAI	PIEATKGRH	LIFCHSKKKC	1400
DELAAKLTGL	GLNAVAYYRG	LQSVIPPPIG	DVVVAITDAL	MIGFTGDFDS	1450
VIDQNTICVTQ	TVDFSLDPTF	TIEITTVPOD	AVSRSQRRGR	TGRGRSGIYR	1500
FVTPGERPSG	MFDSSVLCEC	YDAGCAWYEL	TPAETSVRLR	AYLNTGRLPV	1550
CQDHLEFWES	VFTGLTHIDA	HFLSQTKQAG	DNFPYLVAHQ	ATVCARAQAP	1600
PPSWDQMAKC	LIRLKPTLHG	PTPLLYRLGA	VQNEVILLHP	ITKYIMACMS	1650
ADLEVVTSTW	VLVGGVLAAL	AAYCLITGVS	VIVGRIILSG	KPAVVPDREV	1700
LYQEFDMEEE	CASQLPYIEQ	GMQLAEQFKQ	KALGLLQATAT	KQAEAAAPVW	1750
ESKWRALETF	WAKHMMNFIS	GIOYLAGLST	LPGNPATIASL	MAFTASITSP	1800
LITQNTILLFN	ILGGWAAQL	APPSAASAFV	GAGIAGA AVG	SIGLGKVLVD	1850
ILAGYGAGVA	GALVAFKVM	GEVPSTEDLV	NLLPAILSPG	ALVGVVCAA	1900

FIG. 14G

SUBSTITUTE SHEET (RULE 26)

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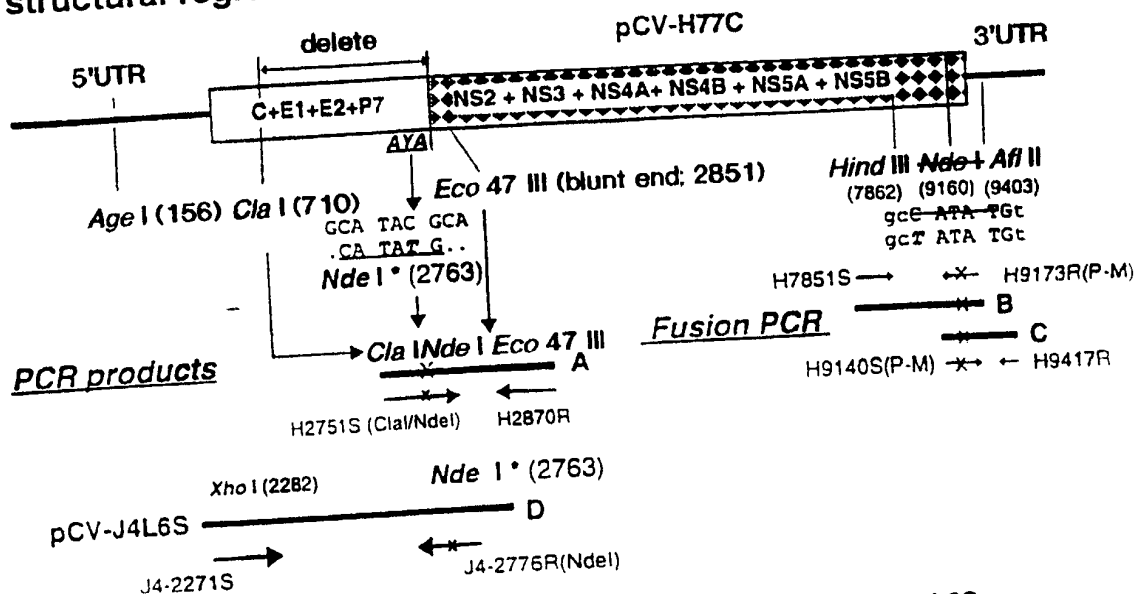
10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
ILRRHVGPGE	GAVQWMNRLI	AFASRGNHVS	PIHYVPESDA	AARVTQILSS	1950
LTTITQLLKRL	HQWINEDCST	PCSGSWLRDV	WDWICTVLTD	FKTWLQSKLL	2000
PRLPGVPFLS	CQRGYKGMWR	GDGIMQTTCP	CGAQIAGHVK	NGSMRIVGPR	2050
TCSNIWHGTF	PINAYTTIGPC	TPSPAPNYSR	ALWRVAAEEY	VEVTRVGDFH	2100
YVIGMTIDNV	KCPCQVPAPF	FFTEVDGVRL	HRYPACKPL	LREDVTFQVG	2150
LNQYLVGSQ	PCEPEPDVTV	LTSMLTDPH	TTAETAKRRL	ARGSPPSLAS	2200
SSASQLSAPS	LKATCTIHHD	SPDADLLEAN	LLWRQEMGN	TIRVESENKV	2250
VILDSFEPLH	AEGDEREISV	AAETILRKSrk	FPSALPIWAR	PDYNPILLES	2300
WKDPDYVPFV	VHGCPLPPTK	APPPIPPRRK	RIVVLITESN	SSALAEATK	2350
TFGSSGSSAV	DSGTATALPD	LASDDGDKGS	DVESYSSMPP	LEGEFGDPDL	2400
SDGSWSTVSE	EASEDVVCCS	MSYTWIGALI	TPCAAEEKSL	PINPLSNSLL	2450
RHNMVYATT	SRSASLRQKK	VTFDRLQVLD	DHYRDVLKEM	KAKASTVKAK	2500
LLSIEEACKL	TPPHSAKSKF	GYGAKDVRNL	SSRAVNHIRS	WEDLLEDTE	2550
TPIDTTIMAK	SEVFCVQPEK	GGRKPARLIV	FPDLGVRVCE	KMALYDVST	2600
LPQAVMGSSY	GFQYSPKQRV	EFLVNIWKS	KCPMGFSYDT	RCFDSTVIES	2650
DIRVEESTYQ	CCDLAPEARQ	AIRSLTERLY	IGGPLINSKG	QNGYRRORA	2700
SGVLTTSCGN	TLTCYLKATA	ACRAAKLQDC	TMLVNGDDL	VICESAGTQE	2750
DAAALRAFTE	AMTRYSAFPG	DPPQPEYDLE	LITSCSSNVS	VAHDASGKRV	2800
YYLTRDPTTP	LARAWEIAR	HTPINSWLG	IIMYAPTLWA	RMILMTHFFS	2850
ILLAQEQLK	ALDOQIYGAC	YSIEPLDLPO	IIERLHGLSA	FTLHSYSPGE	2900
INRVASCLRK	LGVPLRIWR	HRARSVRACL	LSQGGRAATC	GRYLFNWAVER	2950
TKLKLTPIPA	ASQDLGSGWF	VAGYSGGDTY	HSLSRARPRW	FPLCLLLLSV	3000
GVGYLLLENR					3010

FIG. 14H

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#2. Strategy for constructing chimeric clone of HCV (pH77CV-J4)

which contains the nonstructural region of strain H77 and the structural region of strain HC-J4



1. Fragment A, B, C and D ; PCR amplification from pCV-H77C or pCV-J4L6S
 - Fragment A ; additional *Cla* I site, artificial *Nde* I site induced by a single mutation (C→T at nt 2765 of H77C) and authentic *Eco*47 III site
 - Fragment B and C ; eliminated *Nde* I site by a single mutation within the primers (C→T at nt 9158 of H77C) , and fusion PCR with both fragments
 - Fragment D ; artificial *Nde* I site induced by 2 point mutations within the primer (T→A at nt 2762 and C→T at nt 2765 of J4L6S)
2. TA cloning of PCR products
3. Sequence analysis
4. Cloning of Fragment A (*Cla* I-*Eco* 47III) and Fragment B/C (*Hind* III-*Afl* II) with correct sequence into pCV-H77C
5. Complete sequence analysis of new cassette vector [pH77CV], into which the structural regions of different genotypes can be inserted.
6. Cloning of Fragment-Age I/Xho I (cut out from pCV-J4L6S) and Fragment D (*Xho* I-*Nde* I) with correct sequence into the new cassette vector ; 3 piece ligation
7. Complete sequence analysis of 1a+1b chimera [pH77CV-J4]
8. *In vitro* transcription (within 24 hours of inoculation)
9. Percutaneous intrahepatic transfection into chimpanzee

FIG. 15

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CCCAGCCCCC	TGATGGGGGC	GACACTCCAC	CATGAATCAC	TCCCCGTGTA	50
GGAACTACTG	TCTTACGCA	GAAAGCGTCT	AGCCATGGCG	TTAGTATGAG	100
TGTCGTGCAG	CCTCCAGGAC	CCCCCCTCCC	GGGAGAGCCA	TAGTGGTCTG	150
CGGAACCGGT	GAGTACACCG	GAAATGGCCAG	GACGACCGGG	TCCTTTCTTG	200
GATCAACCCG	CTCAATGCCCT	GGAGATTTGG	GCGTGGCCCC	GCGAGACTGC	250
TAGCCGAGTA	GTGTGGGGTC	GCGAAAGGOC	TTGTGGTACT	GCCTGATAGG	300
GTGCTTGCGA	GTGCCCCGGG	AGGTCTCGTA	GACCGTGCAC	CATGAGCAAG	350
AATCCTAAAC	CTCAAAGAAA	AACCAAAGCT	AACACCAACC	GCCGCCCCACA	400
GGACGTCAG	TTCCCCGGCG	GTGGTCAGAT	CGTTGGTGGA	GTTTACCTGT	450
TGCCCCGCGAG	GGGCCCCCAGG	TTGGGTGTGC	GCGCGACTAG	GAAGGCTTCC	500
GAGCGGTGCG	AACCTCGTGG	AAGGCGACAA	CCTATCCCAA	AGGCTCGCCG	550
ACCCGAGGGC	AGGGCCTGGG	CTCAGCCCCG	GTACCCCTTG	CCCCCTCTATG	600
GCAATGAGGG	CCTGGGGTGG	GCAGGATGGC	TCCTGTCAAC	CCGCGGCTCC	650
CGGCTAGTT	GGGGCCCCAC	GGACCCCCCG	CGTAGGTCCG	GTAAGTTGGG	700
TAAGGTCATC	GATACCCCTTA	CATGCGGCTT	CGCCGATCTC	ATGGGGTACA	750
TTCCGCTCGT	CGGCGCCCCC	CTAGGGGGCG	CTGCCAGGGC	CTTGGCACAC	800
GGTGTCCGGG	TTCTGGAGGA	CGGCGTGAAC	TATGCAACAG	GGAAGTTGCC	850
CGGTGCTCT	TTCTCTATCT	TCCTCTTGGC	TCGTCTGTCC	TGTTTGACCA	900
TCCCAGCTTC	CGCTTATGAA	GTCCGCAACG	TGTCCGGGAT	ATACCATGTC	950
ACCAACGACT	GCTCCAATC	AAGCATTTGT	TATGAGGCAG	CGGACGTGAT	1000
CATGCATACT	CCCGGGTGGG	TGCCCCGTGT	TCAGGAGGGT	AACAGCTCCC	1050
GTTCGTGGGT	AGCGCTCACT	CCCACGCTCG	CGGOCAGGAA	TGCCAGCGTC	1100
CCCACTACGA	CAATACGACG	CCACGTGCAC	TTGCTCGTTG	GGACGGCTGC	1150
TTTCTGTCTC	GCTATGTACG	TGGGGGATCT	CTCGGGATCT	ATTTTCTCTG	1200
TCTCCAGCT	GTTCACCTTC	TGGCCTCGCC	GGCATGAGAC	AGTGCAGGAC	1250
TGCAACTGCT	CAATCTATCC	CGGCCATGTA	TCAGGTACAC	GCATGGCTTG	1300
GGATATGATG	ATGAACTGGT	CACCTACAAC	AGCCCTAGTG	GTGTGCCAGT	1350
TGCTCCGGAT	CCCACAAGCT	GTGTTGGACA	TGGTGGGGGG	GGCCCCACTGG	1400
GGAGTCCCTG	CGGGCCTTGC	CTACTATTTCC	ATGGTAGGGA	ACTGGGCTAA	1450
GGTTCTGATT	GTGGCGCTAC	TCTTTGCCCG	CGTTGACGGG	GAGACCCACA	1500
CGACGGGGAG	GGTGGCCGGC	CACACCACCT	CCGGGTTTAC	GTCCCTTTTC	1550
TCATCTGGGG	CGTCTCAGAA	AATCCAGCTT	GTGAATACCA	ACGGCAGCTG	1600
GCACATCAAC	AGGACTGCCC	TAAATTGCAA	TGACTCCCTC	CAAAGTGGGT	1650
TCTTTGCCCG	GCTGTTTTAC	GCACACAAGT	TCAACTCGTC	CGGGTGGCCG	1700
GAGCGCATGG	CCAGCTGCCC	CCCCATTGAC	TGGTTGCCCC	AGGGGTGGGG	1750
CCCCATCACC	TATACTAAGC	CTAACAGCTC	GGATCAGAGG	CCTTATTTGCT	1800

FIG. 16A

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GGCATTACGC	GCCTCGACCG	TGTGGTGTGCG	TACCCGCGTC	GCAGGTGTGT	1850
GGTCCAGTGT	ATTGTTTCAC	CCCAAGCCCT	GTGTGTGGTGG	GGACCACCGA	1900
TGGTTCCGGT	GTCCCTACGT	ATAGCTGGGG	GGAGAATGAG	ACAGACGTGA	1950
TGCTCCTCAA	CAACACGCGT	CCGCCACAAG	GCAACTGGTT	CGGCTGTACA	2000
TGGATGAATA	GTA CTGGGTT	CAC TAAGACG	TGCGGAGGTC	CCCCGTGTAA	2050
CATCGGGGGG	GTCGGTAACC	GCACCTTGAT	CTGCCCCACG	GACTGCTTCC	2100
GGAAGCACCC	CGAGGCTACT	TACACAAAAT	GTGGCTCGGG	GCCCTGGTTG	2150
ACACCTAGGT	GCCTAGTAGA	CTACCCATAC	AGGCTTTGGC	ACTACCCCTG	2200
CACCTCAAT	TTTTCCATCT	TTAAGGTAG	GATGTATGTG	GGGGGCGTGG	2250
AGCACAGGCT	CAATGCCGCA	TGCAATTGGA	CTCGAGGAGA	GCGCTGTAAAC	2300
TTCGAGGACA	GGGATAGGTC	AGAACTCAGC	CCGCTGCTGC	TGTCTACAAC	2350
AGAGTGGCAG	ATACTGCCCT	GTGCTTTCAC	CACCCTACCG	GCTTTATCCA	2400
CTGGTTTGAT	CCATCTCCAT	CAGAACATCG	TGGACGTGCA	ATACTGTAC	2450
GGTGTAGGGT	CAGCGTTTGT	CTCCTTTGCA	ATCAAATGGG	AGTACATCCT	2500
GTTCCTTTTC	CTTCTCCTGG	CAGACGCGCG	CGTGTGTGCC	TGCTGTGGA	2550
TGATGCTGCT	GATAGGCCAG	GCTGAGGCCG	CCTTAGAGAA	CTTGGTGGTC	2600
CTCAATGCGG	CGTCCGTGGC	CGGAGCGCAT	GGTATTCTCT	CCTTTCTTGT	2650
GTTCCTCTGC	GCCGCTGGT	ACATTAAAGG	CAGGCTGGCT	CCTGGGCGCG	2700
CGTATGCTTT	TTATGGCGTA	TGGCCGCTGC	TCTGCTCCT	ACTGGCGTTA	2750
CCACCACGAG	CATATGCACT	GGACACGGAG	GTCGCCCGGT	CGTGTGGCGG	2800
CGTGTCTCTT	GTCGGGTAA	TGGCGCTGAC	TCTGTGCGCA	TATTACAAGC	2850
GCTATATCAG	CTGGTGCATG	TGGTGGCTTC	AGTATTTTCT	GACCAGAGTA	2900
GAAGCGCAAC	TGCACGTGTG	GGTTCCCCC	CTCAACGTCC	GGGGGGGGCG	2950
CGATGCCGTC	ATCTTACTCA	TGTGTGTAGT	ACACCCGACC	CTGGTATTTG	3000
ACATCACCAA	ACTACTCCTG	GCCATCTTGG	GACCCCTTTG	GATTCTTCAA	3050
GCCAGTTTGC	TTAAAGTCCC	CTACTTGGTG	CGCGTTCAAG	GCCTTCTCCG	3100
GATCTGCGCG	CTAGCGCGGA	AGATAGCCGG	AGGTCATTAC	GTGCAAATGG	3150
CCATCATCAA	GTTAGGGGGG	CTTACTGGCA	CCTATGTGTA	TAACCATCTC	3200
ACCCCTCTTC	GAGACTGGGC	GCACAACGGC	CTGCGAGATC	TGGCCGTGGC	3250
TGTGGAACCA	GTGCTCTTCT	CCCGAATGGA	GACCAAGCTC	ATCAAGTGGG	3300
GGGCAGATAC	CGCCGCGTGC	GGTGACATCA	TCAACGGCTT	GCCCCGTCTCT	3350
GCCCCTAGGG	GCCAGGAGAT	ACTGCTTGGG	CCAGCCGACG	GAATGGTCTC	3400
CAAGGGGTGG	AGGTGTCTGG	CGCCCATCAC	GGCGTACGCC	CAGCAGACGA	3450
GAGGCCCTCT	AGGGTGTATA	ATCACCAGCC	TGACTGCCCG	GGACAAAAC	3500
CAAGTGGAGG	GTGAGGTCCA	GATCGTGTCA	ACTGCTACCC	AAACCTTCTT	3550
GGCAACGTGC	ATCAATGGGG	TATGCTGGAC	TGTCTACCAC	GGGGCCGGAA	3600

FIG. 16B

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CGAGGACCAT	CGCATCACCC	AAGGGTCCCTG	TCATCCAGAT	GTATACCAAT	3650
GTGGACCAAG	ACCTTGTGGG	CTGGCCCCGCT	CCTCAAGGTT	CCCCGTCATT	3700
GACACCCCTGT	ACCTGCGGCT	CCTCGGACCT	TTACCTGGTC	ACGAGGCACG	3750
CCGATGTCAT	TCCCCGTGGC	CGCGAGGTG	ATACGAGGGG	TAGCCTGCTT	3800
TCGCCCCGGC	CCATTTCCTA	CTTGAAAGGC	TCCTCGGGGG	GTCCGCTGTT	3850
GTGCCCCGGG	GGACACGGCG	TGGGCTTATT	CAGGGCCGGG	GTGTGCACCC	3900
GTGGAGTGGC	TAAAGCGGTG	GACTTTTATCC	CTGTGGAGAA	CCTAGGGACA	3950
ACCATGAGAT	CCCCGGTGT	CACGGACAAC	TCCTCTCCAC	CAGCAGTGGC	4000
CCAGAGCTTC	CAGGTGGCCC	ACCTGCATGC	TCCACCGGC	AGCGGTAAAG	4050
GCACCAAGGT	CCCGGCTGGG	TACGCAGCCC	AGGGCTACAA	GGTGTGGTGG	4100
CTCAACCCCT	CTGTGTCTGC	AACGCTGGGC	TTTGGTGCCT	ACATGTCCAA	4150
GGCCCATGGG	GTGTATCCTA	ATATCAGGAC	CGGGGTGAGA	ACAATTACCA	4200
CTGGCAGCCC	CATCACGTAC	TCCACCTACG	GCAAGTTCCT	TGCGACGGC	4250
GGGTGCTCAG	GAGGTGCTTA	TGACATAATA	ATTGTGTACG	AGTGCCACTC	4300
CACGGATGCC	ACATCCATCT	TGGGCATCGG	CAGTGTCCCT	GACCAAGCAG	4350
AGACTGCGGG	GGCGAGACTG	GTGTGTCTCG	CCACTGCTAC	CCCTCCGGG	4400
TCCGTCACTG	TGTCCCATCC	TAACATCGAG	GAGGTGTCTC	TGTCCACCAC	4450
CGGAGAGATC	CCCTTTTACG	GCAAGGCTAT	CCCCCTCGAG	GTGATCAAGG	4500
GGGGAAGACA	TCTCATCTTC	TGCCACTCAA	AGAAGAAGTG	CGACGAGCTC	4550
GCCGCGAAGC	TGGTCCGATT	GGGCATCAAT	GCCGTGGCCT	ACTACCGGG	4600
TCTTGACGGT	TCTGTCAATC	CGACCAGCGG	CGATGTGTGTC	GTGCTGTGCA	4650
CCGATGCTCT	CATGACTGGC	TTTACCGGGG	ACTTCGACTC	TGTGATAGAC	4700
TGCAACACGT	GTGTCACTCA	GACAGTCGAT	TTCAGCCTTG	ACCTTACCTT	4750
TACCATTTAG	ACAACCACGC	TCCCCAGGA	TGCTGTCTCC	AGGACTCAAC	4800
GCCGGGGCAG	GACTGGCAGG	GGGAAGCCAG	GCACTCTATAG	ATTGTGGCA	4850
CCGGGGGAGC	GCCCCCTCGG	CATGTTCGAC	TGTCGGTCC	TCTGTGAGTG	4900
CTATGACGGG	GGCTGTGCTT	GGTATGAGCT	CAGGCCCCGC	GAGACTACAG	4950
TTAGGCTACG	AGGTACATG	AACACCCCGG	GGCTTCCCGT	GTGCCAGGAC	5000
CATCTTGAAT	TTTGGGAGGG	CGTCTTTACG	GGCTTCACTC	ATATAGATGC	5050
CCACTTTTTTA	TCCCAGACAA	AGCAGAGTGG	GGAGAACTTT	CCTTACCTGG	5100
TAGCGTACCA	AGCCACCGTG	TGCGCTAGGG	CTCAAGCCCC	TCCCCCATGG	5150
TGGGACCAGA	TGTGGAAGTG	TTTGATCCGC	CTTAAACCCA	CCCTCCATGG	5200
GCCAACACCC	CTGCTATACA	GACTGGGGGC	TGTTTCAAGT	GAAGTCACCC	5250
TGACGCACCC	AATCACCAAA	TACATCATGA	CATGCATGTC	GGCGACCTG	5300
GAGGTGCTCA	CGAGCACCTG	GGTGTGCTGT	GGCGGGTCC	TGGCTGCTCT	5350
GGCCGCGTAT	TGCTGTGCAA	CAGGCTGGGT	GGTCATAGTG	GGCAGGATCG	5400

FIG. 16C

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TCTTGTCCGG	GAAGCCGGCA	ATTATACCTG	ACAGGGAGGT	TCTCTACCAG	5450
GAGTTGGATG	AGATGGAAGA	GTCCTCTCAG	CACTTACCGT	ACATCGAGCA	5500
AGGGATGATG	CTCGCTGAGC	AGTTCAGCA	GAAGGCOCTC	GGCCTCCTGC	5550
AGACCGCGTC	CCGCCATGCA	GAGGTTATCA	CCCCTGCTGT	CCAGACCAAC	5600
TGGCAGAAAC	TGGAGGTCTT	TTEGGCGAAG	CACATGTGGA	ATTTCATCAG	5650
TGGGATACAA	TACTTGGCGG	GCCGTGCAAC	GCTGCTTGGT	AACCCCGOCA	5700
TTCCTTCATT	GATGGCTTTT	ACAGCTGCCG	TCACCAGCCC	ACTAACCCT	5750
GGCCAAACCC	TCTCTTCAAA	CATATTTGGG	GGGTGGGTGG	CTGCCCAGCT	5800
CGCCGCCCCC	GGTGGCGCTA	CTGCCCTTGT	GGGTGCTGGC	CTAGCTGGCG	5850
CCGCCATCGG	CAGCGTTGGA	CTGGGGAAGG	TCTCTGTGGA	CATTCTTGCA	5900
GGGTATGGCG	CGGGCGTGGC	GGGAGCTCTT	GTAGCATTCA	AGATCATGAG	5950
CGGTGAGGTC	CCCTCCACGG	AGGACCTGGT	CAATCTGCTG	CCCGCCATCC	6000
TCTCGCCTGG	AGCCCTTTGTA	GTCGGTGTGG	TCTGCGCAGC	AATACTGGCG	6050
CGGCACGTTG	GCCCCGGCGA	GGGGGCAGTG	CAATGGATGA	ACCGGCTAAT	6100
AGCCTTCGCC	TCCCGGGGGA	ACCATGTTTC	CCCCACGCAC	TACGTGGCGG	6150
AGAGCGATGC	AGCCGCCCCG	GTCACTGCCA	TACTCAGCAG	CCTCACTGTA	6200
ACCCAGCTCC	TGAGGCGACT	GCATCAGTGG	ATAAGCTCGG	AGTGTACCAC	6250
TCCATGCTCC	GGTTCCTGGC	TAAGGGACAT	CTGGGACTGG	ATATGCGAGG	6300
TGCTGAGCGA	CTTTAAGACC	TGGCTGAAAG	CCAAGCTCAT	GCCACAAC TG	6350
CCTGGGATTC	CCTTTGTGTC	CTGCCAGCGC	GGGTATAGGG	GGGTCTGGCG	6400
AGGAGACGGC	ATTATGCACA	CTCGCTGCCA	CTGTGGAGCT	GAGATCACTG	6450
GACATGICAA	AAACGGGACG	ATGAGGATCG	TGGTCCCTAG	GACCTGCAGG	6500
AACATGTGGA	GTGGGACGTT	CCCCATTAAAC	GCTTACACCA	CGGGCCCCCTG	6550
TACTCCCCCT	CCTGCGCCGA	ACTATAAGTT	CGCGCTGTGG	AGGGTGTCTG	6600
CAGAGGAATA	CGTGGAGATA	AGGCGGGTGG	GGGACTTCCA	CTACGTATCG	6650
GGTATGACTA	CTGACAATCT	TAAATGCCCG	TGCCAGATCC	CATCGCCCCGA	6700
ATTTTTCACA	GAATTGGACG	GGGTGCGCCT	ACACAGGTTT	GCGCCCCCTT	6750
GCAAGCCCTT	GCTGCGGGAG	GAGGTATCAT	TCAGAGTAGG	ACTCCACGAG	6800
TACCCGGTGG	GGTGGCAATT	ACCCTGCGAG	CCCGAACCGG	ACGTAGCCGT	6850
GTTCAGCTCC	ATGCTCACTG	ATCCCTCCCA	TATAACAGCA	GAGGCGGCCG	6900
GGAGAAGGTT	GGCGAGAGGG	TCACCCCTTT	CTATGGCCAG	CTCCTCGGCT	6950
AGCCAGCTGT	CCGCTCCATC	TCTCAAGGCA	ACTTGCACCG	CCAACCATGA	7000
CTCCCCTGAC	GCCGAGCTCA	TAGAGGCTAA	CCTCCGTGGG	AGGCAGGAGA	7050
TGGGCGGCCAA	CATCACCAGG	GTTCAGTTCAG	AGAACAAAGT	GGTGATTCTG	7100
GACTCCTTTC	ATCCGCTTGT	GGCAGAGGAG	GATGAGCCGG	AGGTCTCCGT	7150
ACCTGCAGAA	ATTCTGCGGA	AGTCTCGGAG	ATTGCGCCCG	GCCCTGCCCG	7200

FIG. 16D

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TCTGGGCGCG	GCCGGACTAC	AACCCCCCGC	TAGTAGAGAC	GTGGAAAAAG	7250
CCTGACTACG	AACCACCTGT	GGTCCATGGC	TGCCCCGTAC	CACCTCCACG	7300
GTCCCCCTCT	GTGCTCCGCG	CTCGGAAAAA	GCGTACGGTG	GTCCTCACCG	7350
AATCAACCTT	ATCTACTGCC	TTGGCCGAGC	TTGCCACCAA	AAGTTTTTGGC	7400
AGCTCCTCAA	CTTCGGGCAT	TACGGGCGAC	AATAAGACAA	CATCCTCTGA	7450
GCCCCCCCCC	TCTGGCTGCC	CCCCCGACTC	CGAGCTTGAG	TCCTATTCTT	7500
CCATGCCCCC	CCTGGAGGGG	GAGCCTGGGG	ATCCGGATCT	CAGCGACGGG	7550
TCATGGTGCA	CGGTACGTAG	TGGGGCCGAC	ACGGAAGATG	TGCTGTGCTG	7600
CTCAATGTCT	TATTCTTGGA	CAGGCGCACT	CGTCAACCCG	TGCGCTGGGG	7650
AAGAACAATA	ACTGCCCCATC	AACGCACTGA	GCAACTCGTT	GCTACGCCAT	7700
CACAATCTGG	TGTATTCCAC	CACCTCAAGC	AGTGCCTTGC	AAAGGCAGAA	7750
GAAAGTCACA	TTTGACAGAC	TGCAAGTCTT	GGACAGCCAT	TACCAGGACG	7800
TGCTCAAGGA	GGTCAAAGCA	GCGGCGTCAA	AAGTGAAGGC	TAACTTGCTA	7850
TCCGTAGAGG	AAGCTTGCCG	CCTGACGCCC	CCACATTCAG	CCAAATCCAA	7900
GTCTGGCTAT	GGGGCAAAAAG	ACGTCCGTTG	CCATGCCAGA	AAGGCCGTAG	7950
CCCACATCAA	CTCCGTGTGG	AAAGACCTTC	TGGAAGACAG	TGTAACACCA	8000
ATAGACACTA	CCATCATGGC	CAAGAACGAG	GTCTTCTGCG	TTCAGCCCTA	8050
GAAGGGGGGT	CGTAAAGCCG	CTCGTCTCAT	CGTGTTCCCC	GACCTGGGCG	8100
TGCGCGTGTG	CGAGAAGATG	GCCCTGTACG	ACGTGGTTAG	CAAGCTCCCC	8150
CTGGCCGTGA	TGGGAAGCTC	CTACGGATTG	CAATACTCAC	CAGGACAGCG	8200
GGTTGAATTC	CTCGTGCAAG	CGTGGAAATC	CAAGAAGACC	CCGATGGGGT	8250
TCTCGTATGA	TACCCGCTGT	TTTGACTCCA	CAGTCACTGA	GAGCGACATC	8300
CGTACGGAGG	AGGCAATTTA	CCAATGTTGT	GACCTGGACC	CCCAAGCCCC	8350
CGTGGCCATC	AAGTCCCTCA	CTGAGAGGCT	TTATGTTGGG	GGCCCTCTTA	8400
CCAATTCAAG	GGGGGAAAAC	TGCGGCTACC	GCAGGTGCCG	CGCGAGCGGC	8450
GTAATGACAA	CTAGCTGTGG	TAACACCCCTC	ACTTGCTACA	TCAAGGCCCG	8500
GGCAGCCCTGT	CGAGCCGCGAG	GGCTCCAGGA	CTGCACCATG	CTCGTGTGTG	8550
GCGACGACTT	AGTCGTTATC	TGTGAAAGTG	CGGGGGTCCA	GGAGGACGGG	8600
GCGAGCCCTGA	GAGCCCTTCAC	GGAGGCTATG	ACCAGGTACT	CCGCCCCCCC	8650
CGGGGACCCC	CCACAACCAG	AATACGACTT	GGAGCTTATA	ACATCATGCT	8700
CCTCCAACGT	GTCAGTCGCC	CACGACGGCG	CTGGAAAGAG	GGTCTACTAC	8750
CTTACCCGTG	ACCTTACAAC	CCCCCTCGCG	AGAGCCCGGT	GGGAGACAGC	8800
AAGACACACT	CCAGTCAATT	CCTGGCTAGG	CAACATAATC	ATGTTTGGCC	8850
CCACACTGTG	GGCGAGGATG	ATACTGATGA	CCCATTTCTT	TAGCGTCTTC	8900
ATAGCCAGGG	ATCAGCTTGA	ACAGGCTCTT	AACGTGTGAG	TCTACGGAGC	8950
CTGCTACTCC	ATAGAACCAC	TGGATCTACC	TCCAATCAAT	CAAGACTCC	9000

FIG. 16E

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ATGGCCTCAG	CGCATTTTCA	CTCCACAGTT	ACTCTCCAGG	TGAAATCAAT	9050
AGGGTGGCG	CATGCTCAG	AAAACCTGGG	GTCCCGCCCT	TGCGAGCTTG	9100
GAGACACCG	GCCCCGAGCG	TCCGCGCTAG	GCTTCTGTCC	AGAGGAGGCA	9150
GGGCTGCTAT	ATGTGGCAAG	TACCTCTTCA	ACTGGGCAGT	AAGAACAAG	9200
CTCAAACTCA	CTCCAATAGC	GGCCGCTGGC	CGGCTGGACT	TGTCGGGTG	9250
GTTCACGGCT	GGCTACAGCG	GGGGAGACAT	TTATCACAGC	GTGTCTCATG	9300
CCCCGCCCCG	CTGGTCTGG	TTTTGCTAC	TCTGCTCGC	TGCAGGGGTA	9350
GGCATCTACC	TCCTCCCCAA	CCGATCAAGG	TTGGGGTAAA	CACTCGGGC	9400
TCTTAAGCCA	TTTCCGTGTT	TTTTTTTTTT	TTTTTTTTTT	TTTTCTTTT	9450
TTTTTTTCTT	TCCTTTCCTT	CTTTTTTTCC	TTTCTTTTTC	CCCTCTTTAA	9500
TGGTGGCTCC	ATCTTAGCCC	TAGTCACGGC	TAGCTGTGAA	AGGTCCGIGA	9550
GGCGCATGAC	TGCAGAGAGT	GCTGATACTG	GCCTCTCTGC	AGATCATGT	9599

FIG. 16F

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10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
MSTNPKPQRK	TKRNINRRPQ	DVKFFGGGQI	VGGVYLLPRR	GPRLGVRATR	50
KASERSQPRG	RRQPIPKARR	PEGRAWAQPG	YFWPLYGNEG	LGWAGWLLSP	100
RGSRPSWGPT	DPRRRSRNLG	KVIDTLTCGF	ADLMGYIPLV	GAPLGGAARA	150
LAHGVKVLED	GVNYATGNLP	GCSFSIFLLA	LLSCLTIPAS	AYEVRNVSGI	200
YHVINDCSNS	SIVVEAADVI	MHTPGCVPCV	QEGNSSRCW	ALITPTLAARN	250
ASVPTTTIRR	HVDLLVGIAA	FCSAMVVDL	CGSIFLVSQI	FTFSPPRHET	300
VQDCNCSIYP	GHVSGHRMAW	DMMNWSPTT	ALVVSQILLRI	PQAVVDMVAG	350
AHWGVLAGLA	YYSMVGNWAK	VLIVALLFAG	VDGETHTTGR	VAGHTTSGFT	400
SLFSSGASQK	IQLVNTINGSW	HINRTALNEN	DSLQIGFFAA	LFYAHKFNSS	450
GCPERMASCR	PIDWFAQGWG	PITYTKENSS	DQRPYOWHYA	PRPCGVVPAS	500
QVCGPVYCFE	PSPVVVGTTD	RSQVPTYSWG	ENEIDVMLLN	NIRPPOGNWF	550
GCTWMNSTGF	TKTCGGPPCN	IGGVGNRTLI	CPTDCFRKHP	EATYTKOGSG	600
PWLTPRCLVD	YPYRLWHYPC	TLNFSIFKVR	MYVGGVEHRL	NAACNWTIRGE	650
RCNLEDRDRS	ELSPLLLSTT	EWQILPCAFT	TLPALSTGLI	HLHQNTVDVQ	700
YLYGVGSAFV	SFAIKWEYIL	LLFLLLADAR	VCACLWMLL	IAQAEAALEN	750
LVLVNAASVA	GAHGILSFLV	FFCAAWYTKG	RLAPGAAYAF	YGVWPLLLLL	800
LALPPRAYAL	DTEVAASCGG	VVLVGLMALT	LSPYKRYTIS	WCMWWLQYFL	850
TRVEAQLHW	VPPINVRGGR	DAVILLMCV	HPTLVFDITK	LLLAIFGPLW	900
ILQASLLKVP	YFVRVQGLLR	ICALARKIAG	GHYVQMAIHK	LGALTGTIVY	950
NHLITPLRDWA	HNGLRDLAVA	VEPVVFSRME	TKLITWGADT	AACGDIINGL	1000
PVSARRQOEI	LLGPADGMVS	KGWRLAPITT	AYAQOTRGLL	GCIITSLTGR	1050
DKNQVEGEVQ	IVSTATQTFL	ATCINGVCWT	VYHGAGTRTI	ASPKGPIVQM	1100
YTINVDQDLVG	WPAPQGSRSI	TPCTCGSSDL	YLVTRHADVI	PVRRRGDSRG	1150
SLLSPPRISY	LKGSSGGPLL	CPAGHAVGLE	RAAVCTRGVA	KAVDFIPVEN	1200
LGTIMRSPVF	TDNSSPPAVP	QSFOVAHLHA	PTGSGKSTKV	PAAYAAQGYK	1250
VLMVNPVAA	TLGFGAYMSK	AHGVDPNIRT	GVRTTTTGSP	ITYSTYGFEL	1300
ADGGCSGGAY	DIIICDECHS	TDATSIIGIG	TVLDQAETAG	ARLVVLATAT	1350
PPGSVTVSHP	NIEEVALSTT	GEIPFYGKAI	PLEVTKGGRH	LIFCHSKKKK	1400
DELAACLVAL	GINAVAYYRG	LIVSVIPTSG	DVVVSTDAL	MTGFTGDFDS	1450
VIDCNTCVTQ	TVDFSLDPTF	TIETTTLPQD	AVSRITQRRGR	TGRGKPGIYR	1500
FVAPGERPSG	MFDSSVLCEC	YDAGCAWYEL	TPAETTVRLR	AYMNTFGLPV	1550
CQDHLEFWEG	VFTGLTHIDA	HFLSQTKQSG	ENFPYLVAYQ	ATVCARAQAP	1600
PPSWDQMKC	LIRLKPTLHG	PTPLLYRLGA	VQNEVTILHP	ITKYIMTOMS	1650
ADLEVVTSTW	VLVGGVLAAL	AAYCLSTGCV	VIVGRIVLSG	KPAIIPDREV	1700
LYQEFDEMEE	CSQHLFYIEQ	GMMLAEQFKQ	KALGLLQATAS	RHAEVITPAV	1750
QINWQKLEVF	WAKHMMNFIS	GIQYLAGLST	LPGNPATIASL	MAFTAAVTSP	1800
LTTGQTLLEN	ILGGWAAQL	AAFGAATAFV	GAGLAGAAIG	SVGLGKVLVD	1850
ILAGYGAGVA	GALVAFKIMS	GEVPSTEDLV	NLLPAILSEG	ALVGVVCAA	1900

FIG. 166

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H77CV-J4aa Sequence

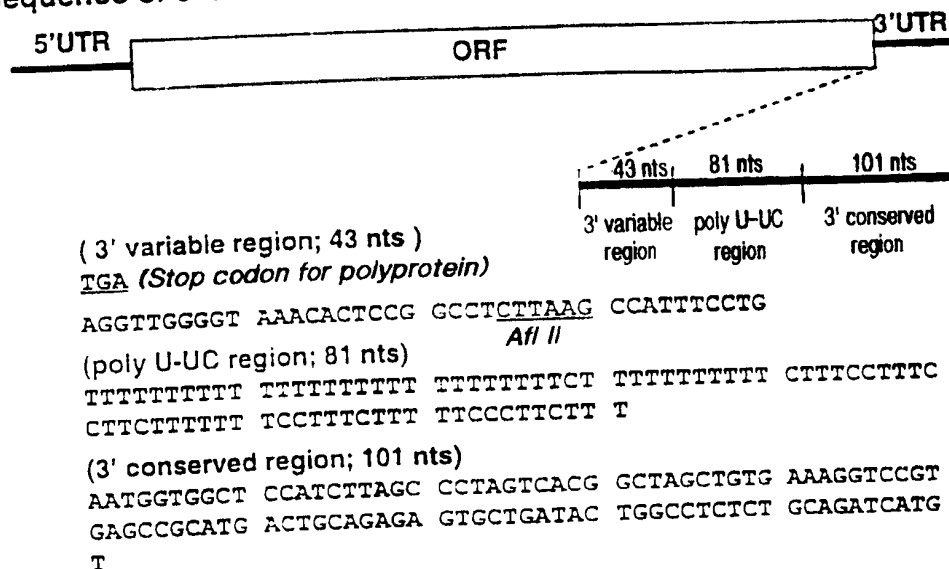
10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
ILRRHVGPGE	GAVQAMNRLI	AFASRGNHVS	PIHYVPESDA	AARVTAILSS	1950
LTVTQLLRRL	HQWISSECTT	PCSGSWLRDI	WDWICEVLSD	FKTWLKAKIM	2000
PQLPGIPFVS	CQRGYRGWR	GDGIMHIRCH	CGAETTGHVK	NGIMRIVGPR	2050
TCRNMWSGTF	PINAYTTGPC	TPLPAFNYKF	ALWRVSAEEY	VEIRRVGDFH	2100
YVSGMITDNL	KCPCQIPSPF	FFTELDGVRL	HRFAPPCKPL	LREEVSFRVG	2150
LHEYFVGSQI	PCEPEPDVAV	LTSMLTDPSH	ITAEAAAGRRL	ARGSPPSMAS	2200
SSASQLSAPS	LKATCTANHD	SPDAELIEAN	LLWRQEMGGN	ITRVESENKV	2250
VILDSFDPLV	AEEDEREVS	PAEILRKSRR	FARALPWWAR	PDYNPPLVET	2300
WKKPDYEPFV	VHGCEPLPPR	SPPVPPPRKK	RIVVLTESTL	STALAEATK	2350
SFGSSSTSGI	TGDNITTSSE	PAPSGCPPDS	DVESYSSMPP	LEGEFGDPDL	2400
SDGSWSTVSS	GADTEDVCC	SMSYSWIGAL	VTPCAAEEQK	LPINALSNSL	2450
LRHHNLVYST	TSRSACQROK	KVTFDRLQVL	DSHYQDVLKE	VKAAASKVKA	2500
NLLSVEEACS	LTPPHSAKSK	FGYGAKDVRC	HARKAVAHIN	SVWKDLLEDG	2550
VTPIDTTIMA	KNEVFVCVQPE	KGGRKPARLI	VFPDLGVRVC	EKMALYDWS	2600
KLPLAVMGSS	YGFOYSPGQR	VEFLVQAWKS	KKTFMEFSYD	TRCFDSTVTE	2650
SDIRTEEATY	QCCDLDPQAR	VAIKSLTERL	YVGGPLINSR	GENCGYRRCR	2700
ASGVLTTSCG	NILTCYIKAR	AACRAAGLQD	CTMLVOGDDL	VVICESAGVQ	2750
EDAASLRAFT	EAMTRYSAAP	GDPPQPEYDL	ELITSCSSNV	SVAHGAGKR	2800
VYILTRDETT	PLARAAWETA	RHTPVNSWLG	NIIMFAPTLW	ARMILMIHFF	2850
SVLIARDQLE	QALNCETYGA	CYSIEPLDLP	PIIQRLHGLS	AFSLHSYSPG	2900
EINRVAACLR	KLGVPPPLRAW	RHRARSVRAR	LLSRGGRAAI	CGKYLEFNWAV	2950
RTKLKLTPIA	AAGRDLDSGW	FTAGYSGGDI	YHSVSHARPR	WFWFCLLLLA	3000
AGVGITYLLN	R				3011

FIG. 16H

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#1a. 3' Deletion mutants of pCV-H77C

Sequence of 3' untranslated region of pCV-H77C



#1a -1. pCV-H77C(-98X) ; 3' 98 nucleotides removed from pCV-H77C

TGAAGGTTGG GGTAACACT CCGGCCTCTT AAGCCATTTC CTGTTTTTTT
 TTTTTTTTTT TTTTTTTTTT TCTTTTTTTT TTTCTTTTCT TTCCTTCTTT
 TTTTCCTTTC TTTTCCCTT CTTTAAT

#1a -2. pCV-H77C(-42X) ; 3' 42 nucleotides removed from pCV-H77C

TGAAGGTTGG GGTAACACT CCGGCCTCTT AAGCCATTTC CTGTTTTTTT
 TTTTTTTTTT TTTTTTTTTT TCTTTTTTTT TTTCTTTTCT TTCCTTCTTT
 TTTTCCTTTC TTTTCCCTT CTTTAATGGT GGCTCCATCT TAGCCCTAGT
 CACGGCTAGC TGTGAAAGGT CCGTGAGCCG CAT

#1a -3. pCV-H77C(X-52) ; All of the 3' UTR sequence, except 3' 49 nucleotides, removed from pCV-H77C

TGAGCCGCAT GACTGCAGAG AGTGCTGATA CTGGCCTCTC TGAGATCAT
 GT

FIG. 17A

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#1a -4. pCV-H77C(X) ; All of the 3' UTR sequence, except 3' 101 nucleotides, removed from pCV-H77C

TGAAATGGTG GCTCCATCTT AGCCCTAGTC ACGGCTAGCT GTGAAAGGTC
CGTGAGCCGC ATGACTGCAG AGAGTGCTGA TACTGGCCTC TCTGCAGATC
ATGT

#1a -5. pCV-H77C(+49X) ; The proximal 49 nucleotides of the 3' conserved region (98 nucleotides ; AAT not included) removed from pCV-H77C

TGAAGGTTGG GGTAAACACT CCGGCCTCTT AAGCCATTTT CTGTTTTTTT
TTTTTTTTTT TTTTTTTTTT TCTTTTTTTT TTTCTTTCCT TTCCTTCTTT
TTTTCCCTTC TTTTCCCTT CTTTAATGCC GCATGACTGC AGAGAGTGCT
GATACTGGCC TCTCTGCAGA TCATGT

#1a -6. pCV-H77C(VR-24) ; First 24 nucleotides of the 3' variable region removed from pCV-H77C

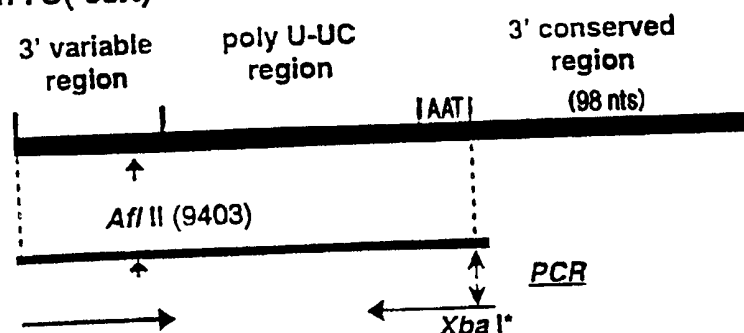
TGACTTAAGC CATTCCTGT TTTTTTTTTT TTTTTTTTTT TTTTTTCTT
TTTTTTTTTC TTCCCTTCC TTCTTTTTTT CCTTCTTTT TCCCTTCTTT
AATGGTGGCT CCATCTTAGC CCTAGTCACG GCTAGCTGTG AAAGGTCCGT
GAGCCGCATG ACTGCAGAGA GTGCTGATAC TGGCCTCTCT GCAGATCATG
T

#1a -7. pCV-H77C(-U/UC) ; Poly U-UC region removed from pCV-H77C

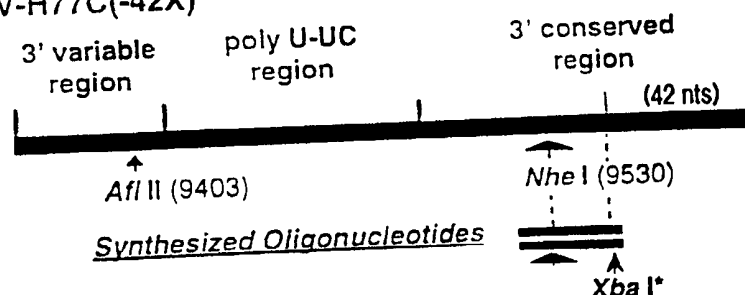
TGAAGGTTGG GGTAAACACT CCGGCCTCTT AAGCCATTTT CTGAATGGTG
GCTCCATCTT AGCCCTAGTC ACGGCTAGCT GTGAAAGGTC CGTGAGCCGC
ATGACTGCAG AGAGTGCTGA TACTGGCCTC TCTGCAGATC ATGT

FIG. 17B

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#1b. Strategy of 3' Deletion mutants**#1b -1. pCV-H77C(-98X)**

1. PCR Amplification
2. Purification of PCR products
3. Digestion with *Afl* II and *Xba* I
4. Cloning of *Afl* II /*Xba* I fragment into pCV-H77C
5. Complete sequence analysis
6. in vitro transcription (within 24 hours of inoculation)
7. Percutaneous intrahepatic transfection into chimpanzee ; 11/26/97 and 12/17/97
8. Result : Negative (No replication)

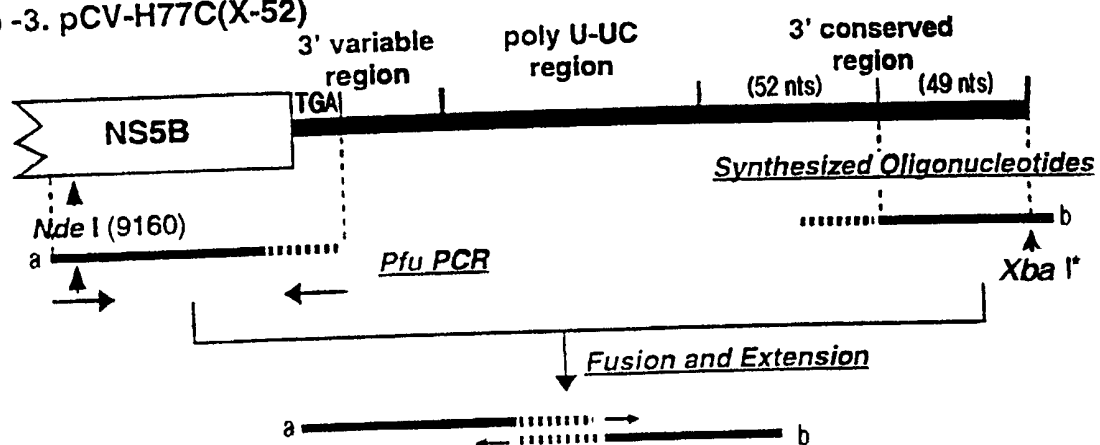
#1b -2. pCV-H77C(-42X)

1. Synthesis of oligonucleotides (sense and anti-sense)
2. Hybridization of oligonucleotides
3. Digestion with *Nhe* I and *Xba* I
4. Cloning of *Nhe* I /*Xba* I fragment into pG9-KL26 (3' UTR of H77C)
5. Sequence analysis
6. Cloning of 3' UTR (-42X) [*Afl* II /*Xba* I fragment] into pCV-H77C
7. Complete sequence analysis
8. in vitro transcription (within 24 hours of inoculation)
9. Percutaneous intrahepatic transfection into chimpanzee (Schedule; 1/22/98, 2/5/98)

FIG. 17C

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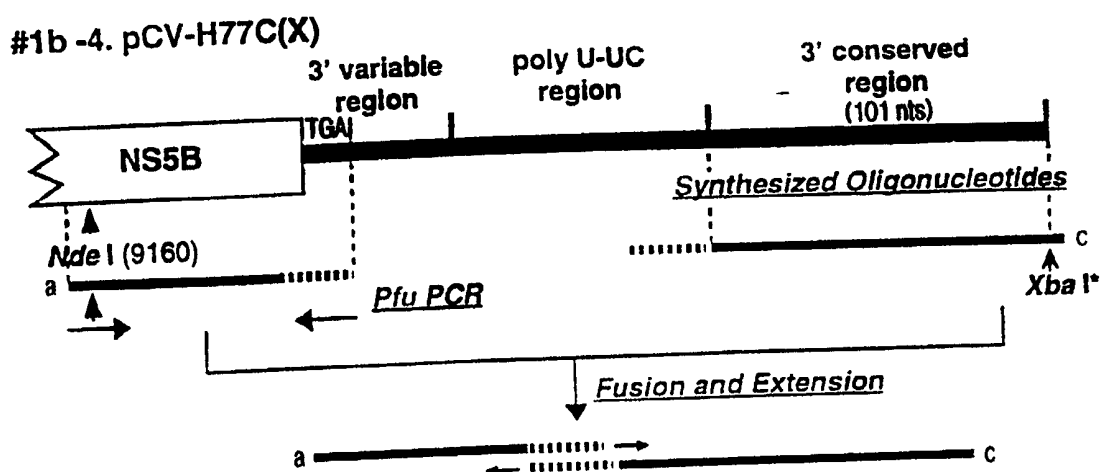
#1b -3. pCV-H77C(X-52)



1. Fragment a ; *Pfu* PCR amplification and purification
2. Fragment b ; Synthesized oligonucleotides (anti-sense)
3. Fusion and extension
4. TA cloning
5. Sequence analysis
6. Cloning *Nde* I-*Xba* I fragment with correct sequence into pCV-H77C
7. Complete sequence analysis
8. *In vitro* transcription (within 24 hours of inoculation)
9. Percutaneous intrahepatic transfection into chimpanzee

FIG. 17D

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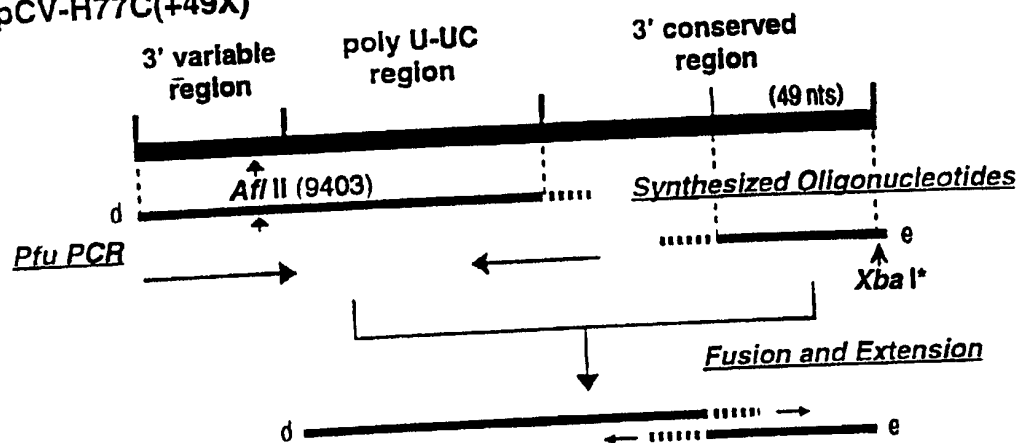


1. Fragment a ; *Pfu* PCR amplification and purification
2. Fragment c ; Synthesized oligonucleotides (anti-sense)
3. Fusion and extension
4. TA cloning
5. Sequence analysis
6. Cloning *Nde* I-*Xba* I fragment with correct sequence into pCV-H77C
7. Complete sequence analysis
8. *In vitro* transcription (within 24 hours of inoculation)
9. Percutaneous intrahepatic transfection into chimpanzee

FIG. 17E

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#1b -5. pCV-H77C(+49X)

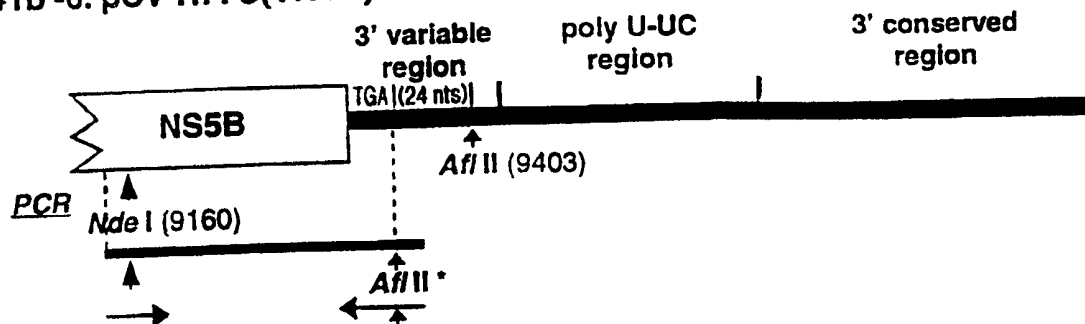


1. Fragment d ; *Pfu* PCR amplification and purification
2. Fragment e ; Synthesized oligonucleotides (anti-sense)
3. Fusion and extension
4. TA cloning
5. Sequence analysis
6. Cloning *Afl* II-*Xba* I fragment with correct sequence into pCV-H77C
7. Complete sequence analysis
8. *In vitro* transcription (within 24 hours of inoculation)
9. Percutaneous intrahepatic transfection into chimpanzee

FIG. 17F

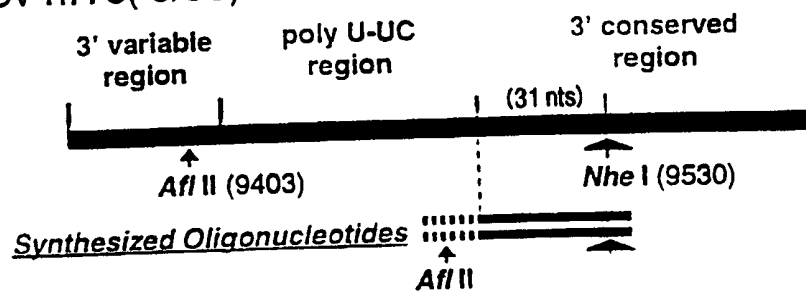
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#1b -6. pCV-H77C(VR-24)



1. PCR Amplification
2. Purification of PCR products
3. Digestion with *Nde* I and *Afl* I
4. Cloning of *Nde* I / *Afl* II fragment into pCV-H77C
5. Complete sequence analysis
6. in vitro transcription (within 24 hours of inoculation)
7. Percutaneous intrahepatic transfection into chimpanzee

#1b -7. pCV-H77C(-U/UC)



1. Synthesis of oligonucleotides (sense and anti-sense)
2. Hybridization of oligonucleotides
3. Digestion with *Afl* II and *Nhe* I
4. Cloning of *Afl* II and *Nhe* I fragment into pG9-KL26
5. Sequence analysis
6. Cloning of 3' UTR (-poly U-UC) [*Afl* II / *Xba* I fragment] into pCV-H77C
7. Complete sequence analysis
8. in vitro transcription (within 24 hours of inoculation)
9. Percutaneous intrahepatic transfection into chimpanzee

FIG. 17G

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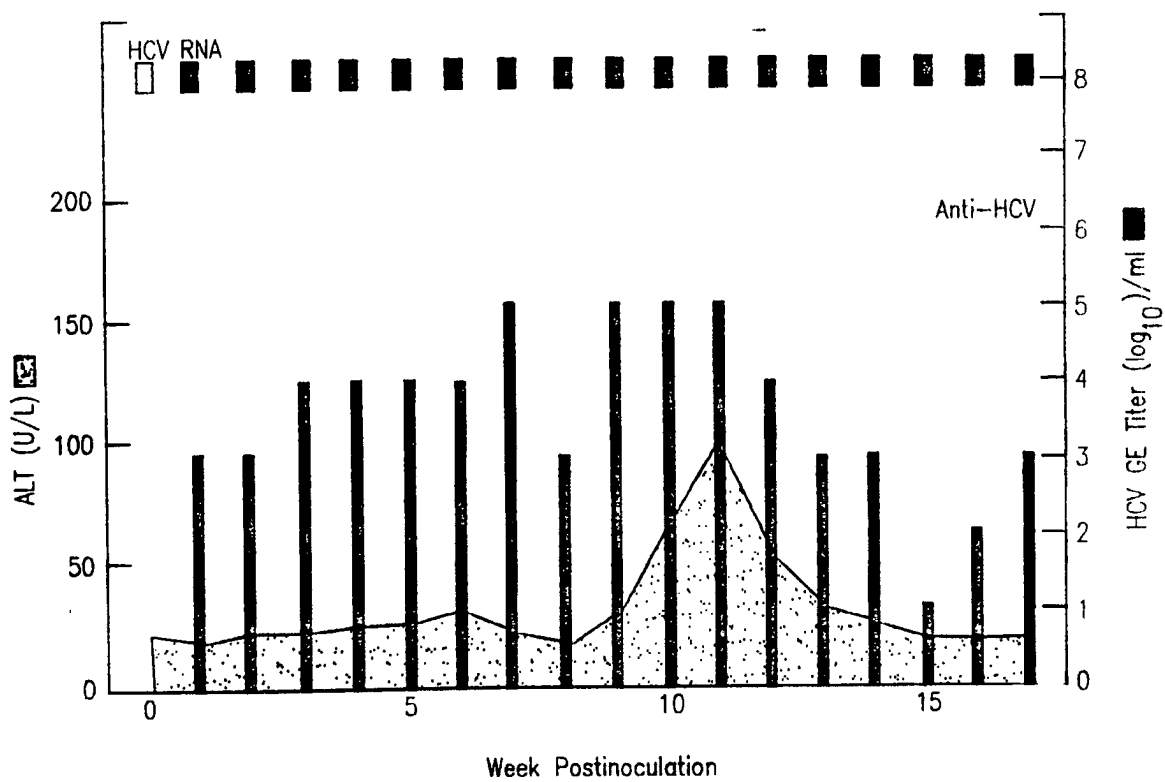


FIG. 18A

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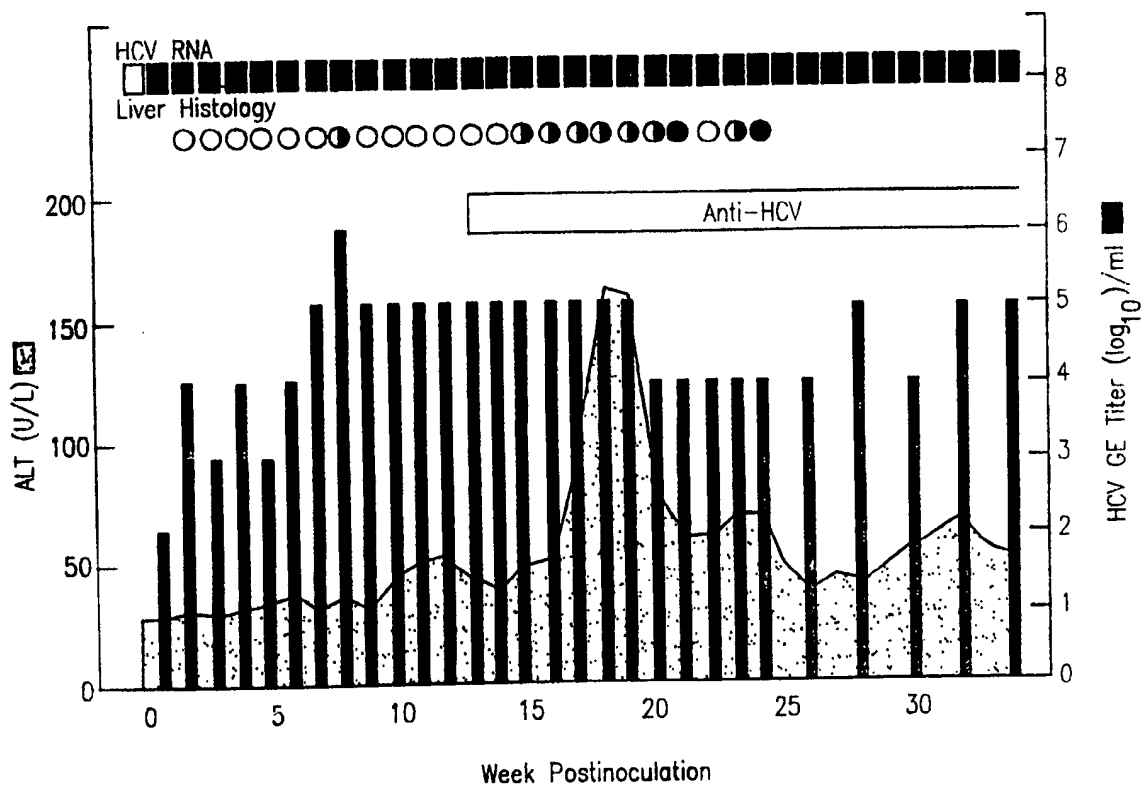


FIG. 18B